GLYCOGENOSIS TYPE II

CLONING AND CHARACTERIZATION OF THE HUMAN LYSOSOMAL α-GLUCOSIDASE GENE

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KLONERING EN KARAKTERISERING VAN HET HUMANE LYSOSOMAAL α-GLUCOSIDASE GEN

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Voor mijn ouders
Aan Henk

Contents

Objectives

Glycogenosis type II is a lysosomal storage disorder. Characteristic features are heart failure and generalized muscle weakness. The disease is caused by the inherited deficiency of acid α-glucosidase, the enzyme responsible for the degradation of lysosomal glycogen.

The aim of the work described in this thesis was to isolate and decipher the genetic code for acid α-glucosidase, and to study the relation between enzyme structure and function.

CHAPTER 1

Cells, lysosomes, and protein sorting

1.1 The cell and its compartments

In unicellular organisms the cell performs all the necessary functions for survival and reproduction, but in multicellular organisms cells have become specialized (differentiated) for certain tasks, such as contraction in muscle cells, and conduction of electrical signals in nerve cells. The development of complex organisms is realized by cell division and differentiation. These processes are driven by the sequential action of multiple factors, and require a continuous synthesis and degradation of proteins. Once cells have reached their end-stage of differentiation, communication with neighbouring cells and reaction to a changing environment are still imperative for proper functioning.

In prokaryotes, the DNA duplication, the protein synthesis and degradation, and the energy fixation take place in the cytoplasm. The compartmentalization in eukaryotes has distributed these basic processes over several cell organelles, which allows the cell to perform more complex functions.

The nucleus contains the genetic material, in which the information for the production of cellular and extracellular proteins is imprinted. The mitochondria are involved in the generation of energy-bearing molecules (ATP). Several enzymes with specialized metabolic tasks are localized in the peroxisomes. The endoplasmic reticulum and the Golgi complex are the main sites for synthesis and processing of (glyco)proteins destined for the cellular membranes, the lysosomes, and the secretory pathway. Numerous transport vesicles are involved in shuttling components from one compartment to the other.

The lysosomes have an important function in the degradation of cellular and extracellular macromolecular components. The low molecular weight constituents are re-utilized. Given the vast array and molecular complexity of the components to be degraded, lysosomes are equipped with a large variety of hydrolytic enzymes. In addition, enzyme activators, stabilizers and membrane-transporters for export of degradation products are members of the family of lysosomal proteins. The absence or improper action of one of these lysosomal proteins may prevent the degradation of particular macromolecules, and causes their accumulation. Eventually the metabolic and mechanical function of the cell will be disturbed. At present, over thirty lysosomal protein defects have been assigned to the corresponding disorders. The lysosomal storage disorders are heterogeneous with respect to the clinical presentation, and the extent and variety of storage material (Scriver et al., 1989).

This thesis deals with one of the lysosomal storage disorders, i.e. glycogenosis type II (Pompe disease). The disease is caused by the inherited deficiency of acid α-

glucosidase, the enzyme responsible for the degradation of lysosomal glycogen. The experimental work is focused on the cloning and characterization of the gene coding for acid α-glucosidase. The inheritance of mutant and polymorphic alleles is studied in the Caucasian and Asian population. The work has revealed some unexpected features concerning the structure, transport, and function of acid α-glucosidase. Mutant phenotypes of acid α-glucosidase are often related to abnormalities in enzyme realization and organelle specific targeting. The following paragraphs of this chapter contain more general information on the latter topics.

1.2 The mobilization of genetic information

The genetic information is exported from the nucleus into the cytoplasm in the form of mRNA. After transcription, introns are spliced out before the mRNA leaves the nucleus via the pores. In the cytoplasm, the mRNA binds to ribosomes and is translated into protein, starting at the first AUG-triplet coding for methionine. This AUG-codon is part of a consensus sequence for translation-initiation (Kozak, 1987).

As translation proceeds the nascent protein emerging from the ribosome folds into its proper secondary and tertiary structure. Since all translation takes place in the cytoplasm, it follows that proteins must have a built in "address" that ensures correct transportation to such diverse destinations as the nucleus, the mitochondria, the lysosomes and the extracellular space. The proteins are transported across, or are integrated into membranes without interrupting the integrity of the proteins, the membranes, and the organelles. Some of these import mechanisms and their corresponding signals are discussed below.

1.3 Import into the endoplasmic reticulum

Import into the membrane or the lumen of the endoplasmic reticulum is required for the following groups of proteins (Pfeffer and Rothman, 1987; see for review also Rapoport, 1990).

1) Secretory proteins and (integral) plasma membrane proteins.
2) Soluble and membrane bound lysosomal proteins.
3) Proteins destined for secretory storage vesicles (e.g. granules in mast cells).
4) Resident proteins of the organelles belonging to the intracellular traffic route (endoplasmic reticulum, Golgi complex, endosomes, etc.).

Entry into the endoplasmic reticulum is mediated by a characteristic N-terminal extension, the so-called signal sequence. Signal sequences of different proteins share little primary sequence homology, but the overall design is conserved. Typically, a stretch of 15-30 amino acids is present which can be divided into an N-terminal, positively charged n-region, followed by an apolar h-region, and a c-region with (or

without) a cleavage site for signal peptidase (Watson, 1984; Von Heijne, 1986; 1988; Gierasch, 1989). Directly after synthesis of the signal peptide, the RNA-ribosome complex becomes associated with the endoplasmic reticulum. This association is mediated by the signal recognition particle (SRP), which binds to the signal peptide and the ribosome (Walter et al., 1984). The SRP then becomes attached to an integral membrane protein of the endoplasmic reticulum, the "docking" protein or SRP-receptor (Meyer et al., 1982; Gilmore et al., 1982; Tajima et al., 1986). After docking, the signal peptide interacts with its receptor (SSR) (Wiedmann et al., 1987). The SRP is released, and is free for another recognition-attachment cycle.

The SRP has both RNA and proteins as building blocks. The RNA has homology with human Alu-repeat sequences (Walter and Blobel 1982). Four proteins are contained within the SRP: a 19 kD and a 54 kD polypeptide, and two heterodimers, composed of 9 and 14 kD polypeptides, and of 68 and 72 kD polypeptides, respectively (Walter and Blobel, 1980; Scoulica et al., 1987). By selective inactivation of one or more of the polypeptides the following functional assignments could be made (Siegel and Walter, 1985; 1988). The 9/14 kD heterodimer is involved in the in vitro elongation arrest, while the 68/72 kD heterodimer has a function in the promotion of translocation, probably by interacting with the SRP-receptor. The 54 kD polypeptide is involved in the signal peptide recognition in the cytoplasm (Krieg et al., 1986; Kurzchalia et al., 1986). The 19 kD protein seems to be required for association of the 54 kD protein with the SRP. The RNA molecule is thought to function as a scaffolding on which the polypeptides are mounted (Siegel and Walter, 1986).

It was found that import into the endoplasmic reticulum was stimulated by constitutively expressed hsp70 heat shock-related proteins (Chirico et al., 1988; Deshaies et al., 1988; Beckmann et al., 1990). The exact nature of interaction has not yet been established, but maintenance of an import-competent conformation was suggested, analogous to mitochondrial import.

The signal peptide receptor (SSR) is an integral membrane protein of approximately 35 kD which presumably situates the nascent protein near the import site (Wiedmann et al., 1987). Translocation across the membrane takes place co-translationally. A model has been proposed in which the signal peptide inserts into the membrane in a loop-like fashion, the N-terminus facing the cytoplasm and the C-terminal end facing the lumen (Engelman et al., 1981; Wickner and Lodish, 1985; Shaw et al., 1988). When the peptide chain is elongated and the cleavage site at the C-terminal part of the signal sequence is exposed, the signal peptide is cleaved off by the signal peptidase complex.

The signal peptidase complex consists of six polypeptides at the C-terminal end of the protein (Evans et al., 1986). Little is known as yet about the exact functions of the complex, but a role in the actual translocation machinery cannot be excluded (Cioffi

et al., 1989).

During translocation, oligosaccharide precursors are attached to asparagine residues in the conformation Asn-X-Thr/Ser via en block transfer from dolichol pyrophosphate (N-glycosylation) (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985). Other modifications of the nascent protein are also accomplished shortly after translocation. These involve the formation of disulfide bridges (Bergman and Kuehl, 1979), attachment of phospholipid membrane anchors (Ferguson and Williams, 1988), and folding into the right conformation. Oligomerization and complex assembly may take several hours (Hurtley and Helenius, 1989).

1.4 Export from the endoplasmic reticulum

It has become increasingly clear that transport from the endoplasmic reticulum to the Golgi is highly regulated (Rose and Doms, 1988; Lodish, 1988). Different proteins exit the endoplasmic reticulum at different rates, and others may never leave. Some of the signals involved have been elucidated, and these will be discussed.

There is evidence that peptides and proteins exit the endoplasmic reticulum just by "default". For instance, acylated tripeptides, not containing any transport signals, and trapped into the endoplasmic reticulum by glycosylation, are rapidly and efficiently transported along the secretory pathway (Wieland et al., 1987). This means that resident proteins of the endoplasmic reticulum and the Golgi complex must contain retention signals. Soluble proteins in the endoplasmic reticulum like BiP (Immunoglobulin heavy chain binding protein), GRP94 (glucose-regulated protein 94) and PDI (protein disulfide isomerase), are retained by a consensus sequence at the extreme C-terminal end of the protein, consisting of four amino acids (KDEL in mammals, HDEL in yeast) (Munro and Pelham, 1987; Pelham et al., 1988; Pelham 1989a, 1989b). The same signal is found in peripheral membrane proteins of the endoplasmic reticulum (Fliegel et al., 1989). However, retention by the KDEL-sequence is not absolute since some secretory proteins are greatly retarded, but not retained when the KDEL-sequence is artificially attached to their C-terminus (Zagouras and Rose, 1989).

Transmembrane proteins of the endoplasmic reticulum have different, short, retention signals in their cytoplasmic tail (Pääbo et al., 1987; Nilsson et al., 1989). The reported consensus sequence, consists of two lysines positioned three and four or five residues from the C-terminus (Jackson et al., 1990). Albeit, it is unlikely that all resident endoplasmic proteins have the same retention signals, because of their differential distribution over the different areas of the network, and another retention signal has been reported (Gabathuler and Kvist, 1990).

The most likely mechanism for retention of soluble proteins in the endoplasmic reticulum is that they are recycled back to the endoplasmic reticulum after exit

(Lippincott-Schwartz et al., 1989; Pelham, 1989a, 1989b; Dean and Pelham, 1990). Transport between the endoplasmic reticulum and the Golgi complex is vesicle-mediated (Saraste and Kuismanen, 1984; Groesch et al., 1990). It has been suggested that an intermediate compartment ("salvage compartment") exists between the endoplasmic reticulum and the Golgi complex (Pelham, 1988). The recycling from the Golgi complex to endoplasmic reticulum is microtubule dependent (Lippincott-Schwartz et al., 1990). Recently, a membrane protein that may function as a KDEL receptor has been identified in mammalian cells, using anti-idiotype antibodies (Vaux et al., 1990). The yeast HDEL-receptor was characterized using mutants defective in endoplasmic reticulum retention (reviewed by Warren, 1990).

Correct folding and assembly seems to be obligatory for proteins not containing the KDEL sequence to be able to leave the endoplasmic reticulum (Lodish, 1988; Rose and Doms, 1988; Hurtley and Helenius, 1989; Pelham, 1989a, 1989b). Abnormal or mutated proteins are often retained in the endoplasmic reticulum. This is for instance the case in some forms of hexosaminidase A deficiency (Proia and Neufeld, 1982; Zokaeem et al., 1987), in glycogenosis type II (Reuser et al., 1987), and in Gaucher disease (Willemsen et al., 1987). For apolipoprotein B, it has been suggested that retention is a means of controlling the level of expression (Davis et al., 1990). Another interesting example is the α-chain of the T-cell receptor (TCR). When the α-chain of this complex is overexpressed in transfected cells, and is unable to form complexes with the other subunits of the TCR, the peptide is rapidly degraded. A small C-terminal peptide sequence seems to be instrumental in this degradation. (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1989, 1990). Thus it appears that a quality control exists at the threshold of the endoplasmic reticulum (see for review Klausner and Sitia, 1990).

The mechanism of retention of non-KDEL containing proteins in the endoplasmic reticulum is not exactly known, but association with resident proteins has been observed in several cases (reviewed by Pelham, 1989a, 1989b). One such protein is BiP, which was first identified as the Ig-heavy chain binding protein, but at present it is known to associate also with other newly synthesized proteins in the endoplasmic reticulum. Egasyn, another soluble lumenal protein, complexes with the lysosomal enzyme ß-glucuronidase, thus causing 10-50% of the total ß-glucuronidase to be retained in the microsomes of rat liver cells instead of being transported to the lysosomes (Medda et al., 1989). Since abnormal proteins tend to aggregate into insoluble complexes in the endoplasmic reticulum lumen, it is not excluded that the size of the complexes precludes vesicular export (Hurtley and Helenius, 1989). In conclusion, exit of proteins from the endoplasmic reticulum depends on both retention and transport signals contained in the protein.

1.5 The Golgi complex

The next compartment in the transport pathway of lysosomal, secretory and plasma membrane proteins is the Golgi complex. It consists of a stack of 3-8 cisternae, which communicate via small non-clathrin coated vesicles. Biochemically, at least three different compartments are recognized: *cis*-, *medial*- and *trans*-Golgi (Dunphy and Rothman, 1985). Entry of proteins occurs at the *cis*-side, and exit is at the *trans*-side or TGN (*trans*-Golgi network). Processes taking place in the Golgi complex involve the modification of N-terminal glycosidic chains, O-linked glycosylation, synthesis of glycosaminoglycans, sulfation, and the phosphorylation of lysosomal proteins (Farquhar, 1985). The enzymes of N-glycosidic modification are localized in order of action from *cis*- to *trans*-Golgi. Mannosidase I is present in the *cis*-Golgi cisternae, the medial Golgi contains mannosidase II and GlcNAc-transferase I, while the terminal (fucosyl-, and galactosyl-) transferases are located in the *trans*-subcompartment (Farquhar, 1985; Kornfeld and Kornfeld, 1985). The *cis*-Golgi compartment is also the site where lysosomal enzymes obtain the mannose 6-phosphate recognition marker (Pohlmann et al., 1982). Acquisition of mannose 6-phosphate limits further processing of the carbohydrate chain, which then remains of the high mannose-type (Kornfeld and Kornfeld, 1985). The unphosphorylated carbohydrate chains are processed to bi- tri- and tetra-antennary structures during further transport through the following compartments of the Golgi complex (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985).

The mannose 6-phosphate marker is added in two steps. First, α-N-acetyl-glucosamine 1-phosphate is transferred from UDP-N-acetylglucosamine to a (pen)ultimate mannose residue of a high-mannose unit attached to a lysosomal enzyme (Reitman and Kornfeld, 1981). Secondly, the phosphodiester is processed to a phosphomono-ester by removal of the α-N-acetylglucosamine (Waheed et al., 1981). It has been shown that these steps are mediated by different enzymes; a transferase, and a diesterase, respectively. The transferase is deficient in patients with I-cell disease, and cells from these patients secrete their soluble lysosomal enzymes. The enzyme has - apart from a catalytic site - a separate functional domain for the recognition of lysosomal enzymes (see for review Von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989; Kornfeld, 1990).

Since no primary sequence homology has been found among the cloned sequences of soluble lysosomal enzymes, it seems that the specific recognition site is formed by a discontinuous amino acid motive. This is substantiated by the fact that heat-denatured lysosomal proteins are no substrates for the phosporylating enzyme. For cathepsin D it has been demonstrated that the recognition site consists of two discontinuous amino acid stretches (Baranski et al., 1990; Kornfeld, 1990). This emphasizes that a specific conformation is essential for cellular transport.

Transport from one cisterna to the next is vesicle-mediated. This ATP-requiring transport system has been reconstituted in vitro, and many aspects have been studied (Fries and Rothman, 1980; Rothman and Orci, 1990). The coated vesicles do not contain clathrin (Orci et al., 1986) and their fusion with the target membrane requires GTP-binding proteins (Melançon et al., 1987), fatty acylation (Glick and Rothman, 1987; Pfanner et al., 1990), an N-ethyl-maleimide sensitive factor (NSF) (Malhotra et al., 1988), an integral membrane receptor and additional soluble proteins (Weidman et al., 1989). Vesicle budding has been studied less well, but ATP and fatty acylation are required (Pfanner et al., 1989). NSF has been purified from Chinese hamster ovary cells (Block et al., 1988), and its gene was cloned and sequenced (Wilson et al., 1989). NSF was found to be homologous to the yeast SEC 18-gene product, which is essential for yeast cell growth and which is involved in the vesicle-mediated transport between the endoplasmic reticulum and the Golgi complex (Eakle et al., 1988). Subsequently, it was shown that the same factor is also a critical component in the vesicular transport between the endoplasmic reticulum and the Golgi stacks of mammalian cells (Beckers et al., 1989). Furthermore, NSF is essential for the fusion of endocytic vesicles after receptor-mediated endocytosis (Diaz et al., 1989). Another protein from yeast cytosol, a 25 kD polypeptide, was shown to function in a prefusion step, both with yeast and mammalian membranes (Wattenberg et al., 1990). This indicates that the fusion process of vesicles with membranes is a conserved mechanism, both between species and between the diverse intracellular membranes. It can be speculated that the specificity of fusion is mediated by receptor molecules which are unique to the different vesicle species and targeting membranes.

Retention of two resident Golgi proteins of the Golgi complex has been attributed to their membrane spanning domains (Machamer and Rose, 1987; Colley et al., 1989; Machamer et al., 1990). No consensus signal has been determined as yet. Analogous to the situation in the endoplasmic reticulum, it is predictable that different signals will exist for the compartmentalization of proteins in distinct Golgi cisternae. Another similarity with the endoplasmic reticulum seems to be the existence of a Golgi-specific quality-control mechanism (Zamoyska and Parnes, 1988).

Exit from the Golgi occurs via the *trans*-Golgi network or reticulum (TGN, TGR). This is the main sorting site of soluble lysosomal and secretory proteins (Farquhar, 1985; Griffith and Simons, 1986). In polarized cells, this may also be the site from which proteins are targeted to different regions of the plasma membrane (apical versus baso-lateral). It is clear, that at this branching point in cellular traffic, transport signals are necessary (Klausner, 1989). For lysosomal targeting, this implies the possession of phosphorylated mannose-residues, which enables binding to the mannose-6 phosphate receptor (MPR) (see for review Von Figura and Hasilik, 1986). Other transport signals are less well defined. In contrast to soluble lysosomal enzymes, lysosomal membrane proteins and proteins associated with the lysosomal

membrane do not require the mannose-phosphate recognition marker for correct targeting. For the highly abundant lysosomal membrane proteins a direct pathway from the TGN to the lysosomes has been suggested (Barriocanal et al., 1986; Green et al., 1987; Croze et al., 1989; Morales et al., 1989), and for accumulation in lysosomes a tyrosine residue is required at a specific position in the endoplasmic tail (Williams and Fukuda, 1990). Lysosomal phosphatase, which behaves like an integral membrane protein till it is solubilized within the lysosome, follows an indirect route via the plasma membrane and the endosomes to the lysosome (Waheed et al., 1988; Gottschalk et al., 1989; Braun et al., 1989). Also for this protein, a tyrosine-containing signal in the cytoplasmic tail is necessary for lysosomal targeting (Peters et al., 1990). For glucocerebrosidase it has been demonstrated that efficient transport to the lysosomes is dependent on complex oligosaccharide formation (Aerts et al., 1986). The enzyme is membrane associated (Mueller and Rosenberg, 1977; Imai, 1985) and does not undergo oligosaccharide phosporylation (Aerts et al., 1988).

1.6 The receptor for lysosomal enzymes

In 1971 the observation of the rapid and selective uptake of "corrective factors" was in fact the first indication that a specific receptor for lysosomal enzymes exists (Neufeld and Cantz, 1971). Subsequently, it was shown that the lysosomal enzymes secreted by I-cell fibroblasts were not recognized by this receptor. Apparently, these enzymes were lacking a recognition marker. When this marker was finally identified as being mannose 6-phosphate, the receptor was named the mannose 6-phosphate receptor (MPR). The multiple lysosomal enzyme deficiencies in I-cell fibroblasts established the importance of the MPR for the intra-cellular sorting of this group of enzymes (see for review, Kornfeld, 1990).

After the MPR had been characterized as a 215 kD integral membrane protein (Sahagian et al., 1981), some murine cell lines were discovered that did not contain this receptor (Gabel et al., 1983). Surprisingly, these cell lines did not exhibit an I-cell phenotype, suggesting the existence of a second receptor. This other receptor turned out to be a much smaller integral membrane protein of 46 kD (Hoflack and Kornfeld, 1985a; 1985b). Based on their differential need for cations, the 215 kD receptor was termed the CIMPR (cation-independent MPR) and the 46 kD receptor the CDMPR (cation-dependent MPR).

The cDNAs for both receptors have been cloned from different mammalian species, including human (Dahms et al., 1987; Morgan et al., 1987; Pohlmann et al., 1987; Lobel et al., 1987; 1988; MacDonald et al., 1988; Oshima et al., 1988; see for review Dahms et al., 1989b). The human CIMPR cDNA codes for a protein of 2491 amino acid residues, including a putative signal sequence (40 amino acid residues), an extracytoplasmic domain of 2264 amino acid residues, followed by a hydrophobic

stretch of 23 residues (the trans membrane domain), and a cytoplasmic domain of 164 amino acid residues. The predicted size of the protein is 275 kD. The CDMPR has a similar organization, with a signal peptide of 20 amino acid residues, an extracytoplasmic domain of 164 residues, a trans-membrane domain of 19 amino acid residues and a cytoplasmic tail of 68 residues (Pohlmann et al., 1987; Oshima et al., 1988).

Sequence analysis revealed that the extracytoplasmic domain of the CIMPR is composed of 15 homologous repeats with an average length of 147 amino acids and a sequence conservation of 14 to 28 %. The extracytoplasmic domain of the CDMPR contains a similar repeat unit (Dahms et al., 1987). This suggests that both receptors have a common evolutionary origin, and that the CIMPR gene has arisen by a number of duplication events.

Subsequent binding studies revealed that 1 mole of mannose 6-phosphate is bound per mole of CDMPR, whereas one mole of CIMPR binds two moles of mannose 6-phosphate (or one mole of any particular lysosomal enzyme) (Tong et al., 1989; Tong and Kornfeld, 1989). Since the CDMPR functions as a dimer (Dahms and Kornfeld, 1989) and the CIMPR probably as a monomer, their binding capacities are similar. This means that only two of the fifteen repeats of the CIMPR have mannose 6-phosphate binding capacity. The function of some of the remaining units must be the binding of IGF-II, since the CIMPR was found to be identical to the IGF-II receptor (Morgan et al., 1987; MacDonald et al., 1988). Although the binding sites are different, interaction of one ligand with the receptor inhibits in part the binding of the other (Tong et al., 1988; MacDonald et al., 1988; Braulke et al., 1988, Waheed et al., 1988). Binding of each ligand has its own characteristic effect on the cell, suggesting the existence of two alternative effector-pathways (Braulke et al., 1990). It is notable, that the MPR of chicken fibroblasts and Xenopus oocytes has no binding site for IGF-II (Clairmont and Czech, 1989), whereas these cells are highly responsive to this growth factor. It is assumed that some of the effects of IGF-II are mediated via the IGF-I receptor. Recently, the α_2-macroglobulin receptor was found to be identical to the LDL-receptor-related protein (Strickland et al., 1990). This suggests that even more receptors may be identified with a dual ligand binding capacity.

Binding of soluble lysosomal enzymes to their receptor(s) takes place in both the biosynthetic and the endocytic pathway. The CDMPR does not take part in endocytosis, whereas the CIMPR is involved in both pathways. Binding of lysosomal proteins to the receptor takes place in the *trans*-Golgi or at the plasma membrane at a neutral pH. Once the receptor-ligand complex has reached the prelysosome/late endosome compartment, the binding is released due to the acidic pH of this compartment (Von Figura and Hasilik, 1986).

1.7 Clathrin-coated vesicles in lysosomal enzyme transport

Clathrin-coated vesicles are involved in both the endocytic and the biosynthetic pathways to the lysosomes. The basic building block of the clathrin-coat is a three-legged structure, named triskelion, composed of three 180 kD (heavy chain) and three 33-36 kD (light chain) molecules (Pearse, 1987). The interaction between clathrin and the cytoplasmic tails of receptors captured in the vesicles is mediated by so-called adaptors. They occur as complexes of 250-300 kD, consisting of two 100 kD polypeptides and two different peptides of 50 and 20 kD, respectively. Two types of adaptors have been identified, HA-I and HA-II (Pearse and Robinson, 1984). The adaptors have one 100 kD polypeptide in common (termed ß-adaptin), whereas the second 100 kD polypeptide differs; α-adaptin in HA-II and γ-adaptin in HA-I. Monoclonal antibodies against α-adaptins (two forms are known: A and C) stain exclusively plasma membrane derived vesicles (Robinson, 1987), whereas γ-adaptin antibodies stain only Golgi-derived clathrin-coated vesicles (Ahle et al., 1988). Antibodies against ß-adaptin react with both types of vesicles. This indicates that the HA-I complex is specifically involved in the biosynthetic pathway, whereas the HA-II complex is characteristic for the endocytic pathway.

The mouse cDNAs coding for α-adaptin A and C have been cloned, and were found to be 84% identical at the amino acid level (Robinson, 1989). Type C α-adaptin is expressed in all tissues whereas type A is only expressed in brain. Also the cDNA coding for ß-adaptin was isolated and sequenced (Ponnambalam et al., 1990). This revealed a total conservation of amino acid sequence of human rat and bovine species. No significant homology with the α-adaptin was found, but similar secondary structures are predicted.

The interaction of the adaptors with different receptors has been investigated. The HA-II adaptor binds to the low density lipoprotein (LDL-)receptor. This binding is inhibited competitively by the cytoplasmic tail of the CIMPR and other receptors (Pearse, 1988). The tyrosine residue a position 807 in the LDL-receptor tail is crucial for rapid internationalization. Tyrosine residues with a similar important function are also present in the cytoplasmic tail of the CIMPR. Altering these two residues by in vitro mutagenesis of cDNA abolished binding to the HA-II adaptor completely. Also the HA-I adaptor binds to the CIMPR tail, but it has, in contrast to the HA-II adaptor, no affinity for the LDL-receptor. Tyrosine residues of the CIMPR tail have been shown not to be essential for HA-I binding (Glickman et al., 1989). These results have been confirmed by expression of mutant CIMPR cDNAs in mouse L-cells (Lobel et al., 1989).

1.8 Targeting to the plasma membrane

Plasma membrane proteins are synthesized like lysosomal and secretory proteins at ribosomes attached to the endoplasmic reticulum. In some membrane bound proteins the signal peptide remains uncleaved, and serves as a membrane anchor. Other membrane bound proteins have a cleaved signal peptide, but are anchored via hydrophobic stretches of 15-20 amino acid residues. These stretches function during translocation as stop-transfer sequences, resulting in proteins with a cytoplasmic C-terminal tail and an N-terminal lumenal domain. Proteins with more than one hydrophobic stretch may cross the membrane several times. The proteins reach the plasma membrane by following the "default" transport route, anchored in the membrane of transport vesicles.

An alternative way for membrane-anchorage is the attachment of glycosyl-phosphatidyl-inositol (GPI) at the C-terminal end shortly after translation (Ferguson and Williams, 1988). Proteins with a GPI-anchor are targeted preferentially to the apical membrane of polarized epithelial cells (Lisanti et al., 1988, 1990; Ali and Evans, 1990)

1.9 Import into other organelles

Mitochondrial import

Mitochondria have a function in the oxidative energy metabolism of eukaryotic cells, and they consist of different compartments. The outer membrane (OM) intermembrane space (IMS), the inner membrane (IM) and the matrix (M) are distinguished. It is an accepted view that mitochondria are derived from endosymbiotic prokaryotes, which during evolution have become specialized cell organelles. They have their own DNA, RNA, and transcription/translation machinery. But, most mitochondrial proteins are encoded in the nucleus, and need to be targeted to their proper compartment within the organelle.

Import into mitochondria is a rather complex process, and several obligatory factors have been identified (see for review, Hartl et al., 1989; Hartl and Neupert, 1990). For instance, a cytosolic 70 kD heat shock-related protein seems to be essential for maintaining the protein in a relatively unfolded state after translation (Murakami et al., 1988). Similar or identical proteins have been implicated for the entry of proteins into the endoplasmic reticulum (Deshaies et al., 1988). The precursors of mitochondrial proteins contain an N-terminal extension of 10-70 amino acid residues, which serves as a recognition and transport signal for import. No sequence homology has been found between the presequences of different mitochondrial proteins but for a general amphiphilic secondary structure with positively charged and hydrophobic amino acids at opposite sides (Roise and Schatz,

1988, Von Heijne, 1988).

A receptor in the mitochondrial membrane guides the precursor of mitochondrial proteins to a general insertion protein (GIP), which facilitates translocation at the contact site between OM and IM. These translocation sites are thought to be proteinaceous channels, since no strong membrane association of precursors can be detected during translocation (Sztul et al., 1989).

Once the targeting signal has passed the membrane, it is cleaved off. Having reached their destination, the matrix proteins then fold and assemble into their active and/or multimeric forms. Most proteins destined for the IM and the IMS have a dual N-terminal signal sequence. The most N-terminal part has the characteristics of a normal mitochondrial import signal and is cleaved off in the matrix after translocation. Thereafter the protein associates with hsp60 (a so-called chaperonin) probably to prevent folding into a non-transportable form. The left over C-terminal part of the presequence is hydrophobic, and serves as an insertion/translocation signal for the IM (Hartl et al., 1989; Hartl and Neupert, 1990).

Nuclear import

The nuclear pores are the channels of communication between the nucleus and the cytoplasm. Preribosomes and RNA leave the nucleus through the pores, and proteins destined for the nucleus enter. Small molecules enter the nucleus by diffusion, but proteins of more than 20-60 kD must possess a specific signal for transport through the pores. The nuclear location signal is formed by a number of basic amino acids arranged in a certain pattern (Kalderon et al., 1984a; 1984b; Siomi et al; 1988; Dang and Lee, 1989; Dingwall et al., 1989; Hatanaka, 1990). The position of the signal does not seem to be very crucial, but exposure of the signal at the surface of the protein is necessary. For instance, the signal of the SV40 large T antigen (PKKKRKV) directs cytoplasmic ß-galactosidase to the nucleus when the signal is attached to the N-terminus of the enzyme (Kalderon et al., 1984b). Other nuclear targeting signals seem to have a more complex structure (Dingwall et al., 1989), and sometimes the signal contains additional information for nuclear localization (Dang and Lee, 1989; Hatanaka, 1990).

Import into peroxisomes

Peroxisomes are organelles bound by a single membrane, which have a variety of metabolic functions in eukaryotic cells. Different cell types have different subsets of activities localized in their peroxisome, and this varies between species. Peroxisomes can be involved in fatty acid ß-oxidation, respiration (based on H_2O_2-forming oxidases and catalase), plasmalogen biosynthesis, the glyoxylate cycle, photorespiration, alcohol oxidation, transaminations and purine and polyamine catabolism. Their abundance varies from few to hundreds per cell, depending on the metabolic state of the cell.

Peroxisomes are generated by division of pre-existing organelles, and are not derived directly from the endoplasmic reticulum (see for reviews Lazarow and Fujiki, 1985; Borst, 1986; Schutgens et al., 1990).

Import of proteins into peroxisomes differs from mitochondrial and endoplasmic reticulum import, because most peroxisomal proteins are synthesized in their mature form, and are not proteolytically processed. Hence, the peroxisomal targeting information must be localized in the mature protein. Indeed, short C-terminal sequences have been identified, which effectuate the transport of proteins to peroxisomes (Gould et al., 1988; Osumi and Fujiki, 1990). It has been demonstrated that the addition of only three conserved amino acids (Ser-Lys-Leu) to the C-terminal end of the cytosolic CAT-enzyme is sufficient for peroxisomal localization (Gould et al., 1989).

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CHAPTER 2

Lysosomal enzymes and their mutations

2.1 The lysosomal storage disorders

Lysosomes and their role in degradative processes in the cell were first described in 1955 (De Duve et al., 1955). The demonstration of acid α-glucosidase deficiency as the primary defect in glycogenosis type II (Hers, 1963) and the localization of this enzyme in lysosomes (Lejeune et al., 1963) hallmarked the first recognition of a lysosomal storage disorder. Subsequently, the deficiency of over thirty lysosomal proteins has been linked to either well-known disorders or to newly discovered syndromes, the most recent ones being ß-mannosidase deficiency (Wenger et al., 1986) and α-N-acetylgalactosaminidase deficiency (Van Diggelen et al., 1987, 1988). Most lysosomal disorders are inherited as autosomal recessive traits, except for α-galactosidase deficiency (Fabry disease) and α-L-iduronate sulfate sulfatase deficiency (Hunter disease) which are X-linked.

The lysosomal diseases can be grouped in several ways, but one of the most common is according to the nature of the storage products. In the mucopolysaccharidoses, the main storage products are glycosaminoglycans. The deficiency of one of the degrading hydrolases, for instance α-L-iduronidase, ß-glucuronidase, heparan-sulfate-sulfatase, N-acetyl-α-D-glucosaminidase, or arylsulfatase B, leads to the accumulation of compounds with non-reducing end sugar moieties characteristic for the deficient enzyme (See for review, Neufeld & Muenzer, 1989). The mucolipidoses comprise sialidosis (N-acetyl-α-neuraminidase-deficiency), I-cell disease and pseudo-Hurler polydystrophy (deficiency of N-acetyl-glucosaminyl phosphotransferase), and mucolipidosis IV. The oligosaccharidoses are due to the deficiency of enzymes involved in the degradation of the carbohydrate chains of glycoproteins, and encompass mannosidosis A and B, fucosidosis, and aspartylglucosaminuria.

Accumulation of sphingolipids (sphingolipidosis) can be caused by the deficiency of a large variety of hydrolases, like hexosaminidase A and B, α- and ß-galactosidase, arylsulfatase A, ceraminidase, sphingomyelinase, glucocerebrosidase, galactocerebrosidase, and N-acetylgalactosaminidase. Some of these hydrolases need activators to perform their function, and the deficiency of a specific activator can lead to the same clinical phenotype as the deficiency of the corresponding hydrolase. Three activator deficiencies have been reported. The G_{M2} activator facilitates the interaction of ß-hexosaminidase A with its lipid substrate, and is deficient in the AB variant of G_{M2}-gangliosidosis (Conzelmann & Sandhoff, 1978; Hechtmann et al., 1982; Hirabayashi et al., 1983). SAP-1 (sphingolipid activator protein, sulfatide activator, G_{M1}-activator, or dispersin) is deficient in a variant form of metachromatic leukodystrophy (Shapiro et al., 1979; Stevens et al., 1981; Hahn et al., 1982). This

activator is involved in the enzymatic activity of arylsulfatase A (Fischer & Jatzkewitz, 1978), ß-galactosidase (Wenger & Inui, 1984) and α-galactosidase A (Li et al., 1985). SAP-2 (glucosylceramidase activator, A1 activator, co-glucosidase, factor P) activates glucocerebrosidase (Ho et al., 1971), galactocerebrosidase, and sphingo-myelinase (Wenger et al., 1982), and is deficient in two rare cases of Gaucher disease (Christomanou et al., 1986; 1989).

Among the sphingolipidoses, the combined deficiency of ß-galactosidase and N-acetyl-neuraminidase (galactosialidosis) has attracted particular attention. The deficiency of a "protective" protein has been demonstrated as the primary defect causing both a decreased stability of ß-galactosidase and a deficiency of N-acetyl-α-neuraminidase (Hoogeveen et al., 1981; D'Azzo et al., 1982; Van Diggelen et al., 1982; Verheyen et al., 1985). Stability of ß-galactosidase is obtained by complex formation between this enzyme and the "protective" protein (Hoogeveen et al., 1983). The nature of the association of the "protective" protein with N-acetyl-neuraminidase has not been fully elucidated, but the purification of N-acetyl-neuraminidase will facilitate studies on this subject (Verheijen et al., 1987; Van der Horst et al., 1989). The "protective" protein was cloned, and was found to have sequence similarity with carboxypeptidase Y from yeast, as well as with the yeast KEX1 gene product, which has carboxypeptidase B-like activity (Galjart et al., 1988). Recently, the "protective" protein was shown to have carboxypeptidase activity (Tranchemontagne et al.,1990), and was suggested to be identical to human cathepsin A (Jackman et al., 1990). Studies with cell lines from patients with galactosialidosis indicate that the "protective" protein is a multifunctional enzyme with esterase, carboxyterminal deamidase, and acid carboxypeptidase activity (Kase et al., 1990).

A relatively new category of lysosomal storage disorders are the transporter-protein-deficiencies like cystinosis (Gahl et al., 1982) and free sialic acid storage disease (Aula et al., 1979; Mancini et al., 1986). In these disorders the end-products of lysosomal degradation accumulate, due to the inability to leave the lysosome. Mancini et al. have demonstrated that glucuronic acid is transported by the same carrier as sialic acid (Mancini et al., 1989).

Pompe disease or glycogenosis type II (acid α-glucosidase deficiency) is the only lysosomal polysaccharide storage disorder. The disease is usually categorized among the glycogen storage disorders, but in this group it is an exception in that the glycogen accumulation is lysosomal rather than cytoplasmic (Baudhuin et al., 1964).

Clinical diversity is often observed within one and the same lysosomal storage disorder. On the other hand, different lysosomal enzyme deficiencies may present in clinically similar patterns. These features complicate the establishment of a correct diagnosis on a purely clinical basis. Therefore, the demonstration of a particular lysosomal enzyme deficiency in cells and/or body fluids of the patient is essential for positive identification of the disorder. For most diseases, the enzyme deficiency can

also be detected in chorionic villi and amniotic fluid cells, offering the possibility of prenatal diagnosis (Kleijer et al., 1986, 1990). The establishment of heterozygosity is much more difficult, due to the large variation of enzyme activity levels among individuals.

The incidence of each individual lysosomal storage disorder varies between 1 in 100,000 to 1 in 300,000. As a group, they are among the most common human inherited diseases. Some diseases occur with high frequency in particular ethnic groups. For instance, the carrier frequency of the infantile form of Tay-Sachs disease is elevated approximately 10 fold (1 in 30) among the Ashkenasi Jewish population when compared to the general population (Petersen et al., 1983). Among the French Canadians the carrier frequency of this disease is similarly high (Andermann et al., 1977). Gaucher disease (deficiency of lysosomal glucocerebrosidase) has been divided in three subtypes on the basis of clinical signs and symptoms. The chronic non-neuronopathic form (type I) has a high incidence among the Ashkenasi Jewish population (estimated carrier frequency 1 in 20) (Matoth et al., 1987), whereas type II and type III disease are rare. In Sweden the Norrbottnian form of Gaucher disease is prevalent (Dreborg et al., 1980). Aspartylglucosaminuria (Beaudet & Thomas, 1989) and Salla disease (Gahl et al., 1989) are almost exclusively confined to the Finnish population.

Reasons to study lysosomal storage disorders are twofold: studying disease processes can reveal aspects of normal processes in the cell, and understanding the disease processes might lead to the development of strategies for prevention or treatment.

It has become evident that mutations leading to disease can exert their effect at all possible levels of lysosomal protein realization. For instance, complete protein deficiency can result from gene deletions or insertions (O'Dowd et al., 1986; Myerowitz & Hogikyan, 1986, 1987; Bernstein et al., 1989; Wilson et al., 1990), and from defective transcription and/or translation (Paw & Neufeld, 1988). Mutant protein precursors can be arrested and degraded in the endoplasmic reticulum (Proia & Neufeld, 1982; Zokaeem et al., 1987; Reuser et al., 1987, Willemsen et al., 1987). Furthermore, they may lack the possibility to acquire the lysosome-targeting signal (mannose 6-phosphate) (Reuser et al., 1987), or are catalytically inactive once the protein has reached the lysosome (Reuser et al., 1987; Willemsen et al., 1987). In some diseases a tentative relation has been established between clinical phenotype, residual enzyme activities and aberrant protein patterns. (Conzelmann et al., 1983; Reuser et al., 1985; O'Dowd et al., 1986; Reuser et al., 1987; Neufeld, 1989).

The dissection of the coding information has been pursued to establish the nature of the mutations at the DNA level. The primary structure of about half of the lysosomal proteins associated with a disease has been unraveled through the cloning of the corresponding human cDNA and/or genomic sequences (Table 1, 2). In some

instances mutations have been discovered, and their effect on the posttranslational processing, transport and catalytic activity of the mutant enzyme protein has been studied. In the following sections some of the results will be discussed, and general conclusions will be drawn.

2.2 Cloning the cDNA's of lysosomal proteins

In Table 1 the lysosomal enzymes are listed for which cDNA clones have been obtained (updated till december 1990).

The isolation of the first cDNA clones coding for lysosomal enzymes was achieved in fairly elaborate ways. A cDNA library was constructed from mRNA enriched in the species of interest. Enrichment was obtained by polysome immunoselection (Myerowitz & Proia, 1984) or by size fractionation of mRNA on a sucrose gradient (Catterall & Leary, 1983; Watson et al., 1985). Fractions of interest were selected by in vitro translation. The proper clones from the resulting cDNA library were then identified by their ability to select mRNA coding for the desired protein. In the case of ß-glucuronidase, kidneys from androgen-stimulated mice or preputial glands from rats were used as mRNA source, because of the high level of expression in these particular organs (Hieber, 1982; Catterall & Leary, 1983; Palmer et al., 1983). Based on the interspecies sequence conservation it has been relatively easy to isolate the human counterpart.

More recently, two other methods have been used with great succes to isolate lysosomal enzyme cDNA clones. One method is to screen a phage expression library in which foreign cDNAs have been inserted into the E. coli ß-galactosidase gene. Phages containing (part of) the correct cDNA express fusion proteins, which can be identified by monospecific antisera. Acid α-glucosidase cDNA has been isolated in this way (Hoefsloot et al., 1988), as has been the majority of other cDNA's coding for lysosomal enzyme proteins (see Table 1 for references). The second method uses oligonucleotides complementary to the cDNA as a probe to screen phage cDNA libraries. The nucleotide sequence of the probes is designed according to the N-terminal amino acid sequence of the protein of interest, or according to the sequences of for instance CNBr or tryptic peptides. Once a cDNA clone has been isolated, the gene structure may be elucidated by isolation and characterization of genomic clones using hybridization to the cDNA.

2.3 Lysosomal protein genes and their promoters

The transcription of a gene is controlled by proteins interacting with the promoter and other regulatory elements. Some genes are expressed only in specialized cells, during a short period of the cell cycle, or at a certain stage of development and

Table 1. Lysosomal enzymes for which human cDNA has been cloned

Lysosomal protein	associated disease	References
-α-glucosidase	Pompe/glycogenosis type II	(1)
Mucopolysaccharidoses		
-α-L-iduronidase	Hurler/MPS I	(2)
-iduronate-sulfate sulfatase	Hunter/MPS II	(3)
-N-acetyl-α-D-glucosamine-6-sulfate-sulfatase	San Fillipo D/MPS III D	(4)
-ß-galactosidase	Morquio B/MPS IV B	(5)
-arylsulfatase B	Maroteaux-Lamy/MPS VI	(6)
-ß-glucuronidase	Sly/MPS VII	(7)
Oligosaccharidoses		
-α-fucosidase	fucosidosis	(8)
-aspartylglucosaminidase	aspartylglucosaminuria	(9)
Sphingolipidoses		
-ß-galactosidase	G_{M1}-gangliosidosis	(5)
-ß-hexosaminidase A (α chain)	G_{M2}-gangliosidosis/Tay-Sachs	(10)
-ß-hexosaminidase A and B (ß chain)	G_{M2}-gangliosidosis/Sandhoff	(11)
-"protective" protein	galactosialidosis	(12)
-α-galactosidase A	ceramidetrihexoside lipidosis/Fabry	(13)
-arylsufatase A	metachromatic leukodystrophy (MLD)	(14)
-glucocerebrosidase	glycosylceramide lipidosis/Gaucher	(15)
-sphingomyelinase	sphingomyelin lipidosis/Niemann Pick A/B	(16)
-α-N-Acetylgalactosaminidase	Schindler disease	(17)
-saposin A,B,C,D	MLD-variant, Gaucher-variant	(18)
-G_{M2}-activator	G_{M2}-gangliosidosis-variant	(19)
Proteases		(20)
-Cathepsin B		(21)
-Cathepsin D		(22)
-Cathepsin M		(23)
-Cathepsin L		
Miscellaneous		(24)
-acid phosphatase		(25)
-h-lamp 1		(26)
-h-lamp 2		

(1) Martiniuk et al., 1986, 1990; Hoefsloot et al., 1988; (2) Scott et al., 1990; (3) Wilson et al., 1990; (4) Robertson et al., 1988; (5) Oshima et al., 1988; Morreau et al., 1989; Yamamoto et al., 1990; (6) Litjens et al., 1989; Schuchman et al., 1990; (7) Guise et al., 1985; Oshima et al., 1987; (8) Fukushima et al., 1985; O'Brien et al., 1987; Occhiodoro et al., 1989; (9) Fisher et al., 1990; (10) Myerowitz & Proia, 1984; Myerowitz et al., 1985; Korneluk et al., 1986; (11) O'Dowd et al., 1985; Korneluk et al., 1986; (12) Galjart et al., 1988; (13) Calhoun et al., 1985; Bishop et al., 1986; Tsuji et al., 1987a; (14) Stein et al., 1989; (15) Ginns et al., 1984; Sorge et al., 1985a; Tsuji et al., 1986; Reiner et al., 1987; (16) Quintern et al., 1989; (17) Tsuji et al., 1989; (18) Dewji et al., 1986, 1987; O'Brien et al., 1988; Nakano et al., 1989; Rorman & Grabowski, 1989; (19) Schröder et al., 1989; (20) Fong et al., 1986; (21) Faust et al., 1985; (22) Fuchs & Gassen, 1989 (23) Joseph et al., 1988 (24) Pohlmann et al., 1988; (25) Viitala et al., 1988; Fukuda et al., 1988; (26) Fukuda et al., 1988

Table 2. Some characteristics of cloned human lysosomal genes and their promoter regions

Gene	Approximate size in kb	Exons			Promoter region			References
		Number	Size range in bp	ATG in exon no.	CG content in %	TATA - CCAAT	Consensus elements	
Acid α-glucosidase	20	20	85-606	2	80	not present	2 AP2-sites	(1)
Acid phosphatase	9	11	83-947	1	59	3 CCAAT incorrectly placed	2 SP1-sites	(2)
ß-Hexosaminidase α	35	14	47-703	1	N.D.	N.D.	N.D.	(3)
ß-Hexosaminidase ß	40-45	14	47-421	1	64	no TATA no CCAAT	4 SP1-sites 2 AP1-sites	(4)
Arylsulfatase	7	2		1	high			(5)
α-Galactosidase	10	7	92-291	1	59	4 TATA 4 CCAAT placing?	1 SP1-site 1 AP1-site 4 "chorion box" enhancers	(6)
Glucuronidase	21	12	85-376	1	62*	1 TATA (correct) no CCAAT*	no SP1-site*	(7)
Glucocerebrosidase	7	11	88-265	2	normal	2 TATA 2 CCAAT correctly placed	no SP1-site	(8)

*Data from mouse gene, D'Amore et al., 1989

(1) Hoefsloot et al., 1990c; (2) Geier et al., 1989; (3) Proia & Soravia, 1987; (4) Neote et al., 1988; Proia, 1988; (5) Kreysing et al., 1990; (6) Quinn et al., 1987; Bishop et al., 1988; (7) Miller et al., 1990 (8) Horowitz et al., 1989

differentiation. The transcription of such genes tends to be strictly regulated by signals from inside and/or outside the cell. In contrast, other genes coding for proteins required for basic cellular processes are constituvely expressed in all cell types. The promoter elements of these "house keeping" genes are different from those of specialized genes. Most house keeping promoters lack the TATA and CCAAT consensus sequences and are GC-rich (Gardiner-Garden & Frommer, 1987).

Lysosomal enzymes are constituvely expressed in all different cell types. But modulation of the expression level of some lysosomal enzymes has been reported. In mouse kidney ß-glucuronidase mRNA levels are increased after treatment with androgen hormones (Palmer et al., 1983). The existence of a regulatory locus (Gus-r) closely linked to the structural gene for glucuronidase has been postulated to explain the different levels of expression in distinct mouse strains (see for review, Paigen, 1979). But this Gus-r locus may as well represent allelic diversity of the promoter region of the ß-glucuronidase sequence. Another interesting example is the lysosomal thiol protease cathepsin L, which becomes the major excreted protein (MEP) upon transformation of mouse fibroblasts (Troen et al., 1987, 1988). Lysosomal glucocerebrosidase seems to have a tissue-specific level of expression (Reiner & Horowitz, 1988; Horowitz et al., 1989).

In Table 2 the cloned genes of human lysosomal proteins are listed, together with some of the characteristic features of the promoter regions. As expected, almost all lysosomal proteins appear to have a "house keeping" gene promoter. Except for the glucocerebrosidase gene, the promoter regions are GC-rich and contain Sp1-binding sites, whereas typical TATA and CCAAT motifs are mostly absent or positioned incorrectly relative to the transcription-initiation site. Sometimes consensus elements for binding of regulatory factors are present in the promoter region. For instance, the promoter of acid α-glucosidase contains two potential AP-2 binding sites, and in the α-galactosidase promoter region a chorion-box enhancer is present. The significance of these elements remains to be determined (see for references, Table 2).

The intron-exon organization of the genes coding for lysosomal proteins is not different from that of other genes. Alternative splicing has been reported for ß-glucuronidase (Oshima et al., 1987), ß-galactosidase (Morreau et al., 1989; Yamamoto et al., 1990), sphingomyelinase (Quintern et al., 1989) and α-N-acetyl-galactosaminidase (Yamauchi et al., 1990). One specific feature shared by at least five lysosomal protein genes is the consensus characterized by a BbvI site that has been found around the translation-initiation site. This sequence was absent in 133 other human non-lysosomal genes (Quinn et al., 1987).

For both glucocerebrosidase (Horowitz et al., 1989) and α-fucosidase (Fowler et al., 1986) the existence of a pseudogene has made the isolation of the correct genomic sequences more difficult. A pseudogene may be instrumental in the generation of mutant alleles. This will be discussed in a following section for

glucocerebrosidase.

2.4 Sequence similarity of lysosomal and other proteins

By sequence comparison, it has become evident that some of the lysosomal proteins are homologous and/or have regions of similarity with other lysosomal or non-lysosomal proteins. The most prominent examples are given in Table 3.

Sequence similarity can evolve in two different ways. One is gene duplication followed by divergence, which can lead to two different gene coding for products with a similar function. The homology of acid α-glucosidase with both sucrase and isomaltase (Hoefsloot et al., 1988) might be explained by this mechanism (see chapter 3), as well as the homology between the two hexosaminidase polypeptide chains (Myerowitz et al., 1985). The common ancestry of the α and ß hexosaminidase genes (localized on chromosomes 15 and 5, respectively) is corroborated by the similar intron-exon organization (Proia, 1988).

Another interesting example of gene duplication is the socalled saposin gene, harbouring the information for four similar activator proteins. When the cDNA for the sphingolipid activator protein SAP-1 was cloned, it appeared to encode a protein four times larger than expected (Dewji et al., 1986, 1987; O'Brien et al., 1988). Sequence analysis revealed an 80% amino acid similarity with rat Sertoli sulfated glycoprotein (Rorman & Grabowski, 1989; Nakano et al., 1989). Moreover, the SAP-1 precursor was found to contain four similar domains, of which the middle two were coding for SAP-1 and SAP-2 (glucosylceramide activator), respectively. Each domain is approximately 80 amino acid residues long. Cystine and proline residues are conserved as well as the glycosylation sites. Also the secondary structure of all four domains was predicted to be similar (O'Brien et al., 1988). The four mature proteins arising from this precursor by proteolytic processing were named saposin A, B, C and D, whereby saposin B is identical to SAP-1 and saposin C to SAP-2 (Morimoto et al., 1988). Saposin D was subsequently purified from spleen, and found to activate acid sphingomyelinase (Morimoto et al., 1988). Purified saposin A was found to have a stimulatory effect on glucosylcerebrosidase and galactosylcerebrosidase, comparable to saposin C (SAP-2) (Morimoto et al., 1989). Since the saposins A to D are encoded in one gene, a double intragenic duplication must have occurred. The signal peptide at the N-terminal end of the protein may have been a more recent addition, or may have been excluded from the duplication event.

The relatively high sequence similarity between α-galactosidase and α-N-acetyl galactosaminidase suggests that also these two genes have arisen from the same ancestral gene (Tsuji et al., 1989; Yamauchi et al., 1990).

When gene duplication event goes awry, pseudogenes may arise. The pseudogene of glucocerebrosidase lies in tandem with the normal gene on chromosome 1

Table 3. Sequence similarities of lysosomal proteins

Lysosomal protein	Sequence similarity	References
acid α-glucosidase	intestinal sucrase/isomaltase	(1)
protective protein	yeast KEX1, yeast carboxypeptidase Y	(2)
glucosamine 6-phosphatase	steroid sulfatase, sea urchin arylsulfatase A, arylsulfatase A and B, iduronate 2-sulfatase	(3,6)
arylsulfatase A	steroid sulfatase, sea urchin arylsulfatase A, iduronate 2-sulfatase, glucosamine 6-phosphatase, arylsulfatase B	(4,6)
arylsulfatase B	steroid sulfatase, arylsulfatase A, glucosamine 6-sulfatase, sea urchin arylsulfatase A, iduronate 2-sulfatase	(5,6)
iduronate 2-sulfatase	steroid sulfatase, arylsulfatase A and B, glucosamine 6-sulfatase, sea urchin arylsulfatase A	(6)
α-N-acetyl galactosaminidase	α-galactosidase	(7)
acid phosphatase	prostatic acid phosphatase	(8)
saposin A,B,C,D	rat Sertoli SGP-1, intragenic homology	(9)
hexosaminidase α-chain	hexosaminidase ß-chain	(10)
glucocerebrosidase	pseudogene	(11)
α-fucosidase	pseudogene	(12)

(1) Hoefsloot et al., 1988; (2) Galjart et al., 1989; (3) Robertson et al., 1988; (4) Stein et al., 1989; (5) Schuchman et al., 1990; (6) Wilson et al., 1990; (7) Tsuji et al., 1989; (8) Geier et al., 1989; (9) O'Brien et al., 1988; Makano et al., 1989; Rorman and Grabowski, 1989; (10) Myerowitz et al., 1985; (11) Horowitz et al., 1989; (12) Fowler et al., 1986.

(Barneveld et al., 1983; Ginns et al., 1985; Horowitz et al., 1989; Zimran et al., 1990a), but has several stop codons in the reading frame. The high degree of homology (96%) indicates a rather recent divergence between the two sequences (Horowitz et al., 1989). The pseudogene for α-fucosidase is located on chromosome 2, whereas the normal gene lies on chromosome 1 (Fowler et al., 1986).

The other way to achieve homology might be exon shuffling by which process functional domains can be acquired. This mechanism might explain the regional similarity of the human sulfatase genes and the existence of conserved sequences between the yeast KEX-1 gene yeast, carboxypeptidase Y, and human protective protein. The same may hold for the sequence similarities of lysosomal acid phosphatase with prostatic acid phosphatase.

2.5 Mutations in lysosomal storage diseases

Studies on the synthesis and post-translational modification of lysosomal proteins in fibroblasts from patients have indicated that lysosomal storage disorders can be caused by a variety of mutations. The identification of the exact genetic aberrations has stressed this point. Even apparently similar protein deficiencies turned out to be caused by different mutations. As anticipated, all possible types of mutations were encountered.

The effect of small or large deletions and insertions can often be predicted, but the ultimate effect of point mutations is difficult to foresee. The amount of mRNA can be estimated by Northern blot analysis, but this parameter has limited value when it concerns the prediction of clinical phenotype. Even when the mRNA steady state level is normal, a complete lack of protein synthesis may be found. Albeit the molecular genetic approach which has been taken in the past ten years to unravel the primary cause of clinical heterogeneity of lysosomal disorders has brought a wealth of new and unexpected information. In the following paragraphs of this chapter the lysosomal storage disorders G_{M2}-gangliosidosis and Gaucher disease are discussed as examples, since they have been studied most extensively.

Hexosaminidase and α-chain mutations

Tay-Sachs disease, caused by the deficiency of the α-subunit of hexosaminidase, has a high frequency (1:30) among the Ashkenasi Jewish population (Peterson et al., 1983). It was expected that one single mutation would prevail, but this prediction appeared only partially true.

Several ß-hexosaminidase isozymes are present in lysosomes. These are designated hexosaminidase A (αß), B ($ß_2$) and S ($α_2$). Their substrate specificities differ, in that hexosaminidase B acts only on the carbohydrate chains of glycoproteins whereas hexosaminidase A hydrolyzes in addition glycosaminoglycans, glycolipids, and with the help of the G_{M2}-activator protein, G_{M2}-ganglioside. Hexosaminidase S has a low catalytic activity for glycosaminoglycans. Two genes are involved in the production of the hexosaminidase isoforms, HEX A and HEX B, coding for the α- and ß-chain, respectively. Mutations in the HEX A gene lead to Tay-Sachs disease or G_{M2}-gangliosidosis variant B, in which condition only hexosaminidase B is functional. Sandhoff disease or G_{M2}-gangliosidosis variant 0 is caused by mutations in the HEX B gene, and is associated with the deficiency of both hexosaminidases A and B. G_{M2}-gangliosidosis variant AB is caused by the deficiency of the G_{M2}-activator protein necessary for hexosaminidase A to act on G_{M2} -ganglioside. Several clinical subtypes of Tay Sachs disease are known, with a different age of onset and severity of symptoms. The infantile-onset form of the disease is the one that occurs frequently among frequently Ashkenasi Jews and French Canadians. Besides, juvenile and adult cases have been

reported (See for review, Sandhoff et al., 1989).

The mutations discovered in the hexosaminidase α-chain are listed in Table 4, together with the combinations of alleles encountered among the different ethnic groups and clinical subtypes. The first remarkable finding concerning infantile Tay-Sachs disease was, that the mutation in the French-Canadian population was different from the one causing the same disease among Ashkenasi Jews. Two unrelated French Canadian patients were found to be homozygous for a deletion in the HEX A gene (Myerowitz & Hogikyan, 1986). A piece of 7.6 kb of DNA was deleted from the 5' end of the gene, starting 2 kb upstream of exon 1, and including the promoter region, exon 1 and part of intron 1 (Myerowitz & Hogikyan, 1987). As expected, no hexosaminidase α-chain mRNA was detected by Northern blot analysis.

The second unexpected finding was, that more than one mutation was responsible for Tay Sachs among Ashkenasi Jews. The most commonly found mutation (70% of the alleles) (Paw et al., 1990b) is an insertion of 4 base pairs in exon 11. The resulting frame shift leads to premature chain termination, 9 base pairs downstream (Myerowitz & Costigan, 1988). No mRNA can be detected by Northern blot analysis, although the transcription rate is normal (Paw & Neufeld, 1988). Apparently, this premature stopcodon gives rise to an unstable mRNA.

A less common mutation was found in 15-30% of the Ashkenasic Jewish Tay-Sachs alleles (Paw et al., 1990b). It concerns a G→C transversion at the 5' border of intron 12 (Myerowitz, 1988; Arpaia et al., 1988; Ohno & Suzuki, 1988a). As a consequence, normal splicing is not possible, and hardly any mature mRNA is formed (Ohno & Suzuki, 1988b).

The third mutation identified among the Ashkenasi Jews is a G→A transition at base pair 805 in exon 7, changing Gly^{269} into Ser (Navon & Proia, 1989; Paw et al., 1989). This mutation however, was only found in adult patients, either in combination with the 4 base pair insertion in exon 11 or with the splice site mutation. In these adult patients mRNA is present (Navon & Proia, 1989) and the α-chain precursor protein is detectable, but the Gly^{269}→Ser mutation causes the α chain to associate poorly with the ß subunit (D'Azzo et al., 1984). The resulting low level of residual hexosaminidase A activity is apparently enough to delay the onset of symptoms to the second or third decade of life. Subsequently, this mutation was also found in homozygous form in non-Ashkenasi Jewish patients with adult G_{M2} -gangliosidosis (Navon et al., 1990).

The three mutations described here do not account for all hexosaminidase α-chain deficiencies in the Ashkenasi Jewish population (Paw et al., 1990b). More recently, it was discovered that also among the French Canadian population more than one mutant allele causes infantile Tay-Sachs disease (Hechtman et al., 1990).

Table 4. Hexosaminidase gene mutations and their effects

Clinical subtype		Genotype	Reference numbers	References
Infantile Tay-Sachs	French Canadians	del 7.6[a] / del 7.6	(1,2)	*(1) Myerowitz and Hogikyan, 1986; 1987; (2) Hechtman et al., 1990; (3) Myerowitz and Costigan, 1988; (4) Arpaia et al., 1988; Myerowitz, 1988; Ohno and Suzuki, 1988a; (5) Ohno and Suzuki, 1988c; (6) Tanaka et al., 1988; (7) Tanaka et al., 1990a; (8) Tanaka et al., 1990b; (9) Nakano et al., 1988; (10) Lau and Neufeld, 1989; (11) Akli et al., 1990; (12) Nakano et al., 1990; (13) Paw et al., 1990a; (14) Navon and Proia, 1989; Paw et al., 1989; (15) Navon et al., 1990*
		del 7.6 / ins 4bp[b]	(2)	
		del 7.6 / ?	(2)	
		ins 4bp / ins 4bp	(2)	
	Ashkenasi Jews	ins 4bp / ins 4bp	(3)	
		ins 4bp / spl12[c]	(3)	
		spl12 / ?	(4)	
	B1-variant	His178[d] / ?	(5,6,7)	
		His178 / His178	(7)	
		His178 / ins 4bp	(7)	
		Cys178[e] / ?	(7)	
	others	Cys420[f] / ?	(8)	
		Lys482[g] / Lys482	(9)	
		del 22aa[h] / del 22aa	(10)	
		spl5[i] / spl5	(11)	
		Gln170[j] / ?	(12)	
Juvenile G_{M2}-gangliosidosis		His504[k] / His504	(13)	
		His499[l] / ins 4bp	(13)	
		? / ?	(13)	
Adult G_{M2}-gangliosidosis	Ashkenasi Jews	Ser269[m] / ins 4bp	(14)	
		Ser269 / spl 12	(14)	
	others	Ser269 / Ser269	(15)	
		Ser269 / ?	(15)	

Notes to Table 4

	Mutation	cDNA[1] position	Genomic position	Amino acid	Effect on mRNA	Effect on protein	Frequency	Reference number
a	del 7.6 kb		promoter -exon 1 - intron 1		absent	absent	most common among French Canadians	(1)
b	4 bp ins	1276	exon 11	codon 426	labile	absent	70% among infantile Ashkenasi Jews	(3)
c	G → C		intron 12 (first bp) splice site		reduced amount, incorrectly spliced	nearly absent	15% among infantile Ashkenasi Jews	(4)
d	G → A	533	exon 5	Arg^{178} → His	normal	catalytically inactive	frequent in B1-variant	(5)
e	C → T	532	exon 5	Arg^{178} → Cys	normal	catalytically inactive	rare (B1-variant)	(7)
f	G → C	1260	exon 11	Trp^{420} → Cys	normal	non-functional	rare	(8)
g	G → A	1444	exon 13	Glu^{482} → Lys	normal	ER retention (insoluble)	rare	(9)
h	del C	1510	exon 13	del 22 amino acids, C-terminal	normal	ER retention (shorter)	rare	(10)
i	G → A	570	exon 5 (last bp) splice site	Leu^{190} → Leu	reduced amount, deletion exon 5	inactive	rare	(11)
j	G → A	509	exon 5	Arg^{170} → Gln	normal	unstable, low specific activity	rare	(12)
k	G → A	1511	exon 13	Arg^{504} → His	normal	association defective (secreted)	rare	(13)
l	G → A	1496	exon 13	Arg^{499} → His	normal	ER retention (abnormal precursor)	rare	(13)
m	G → A	805	exon 7 (last bp)	Gly^{269} → Ser	normal	association defective	frequent in adult G_{M2}-gangliosidosis	(14)

[1]From the first in-frame ATG-codon

Glucocerebrosidase deficiency

Glucosylceramide lipidosis or Gaucher disease is characterized by a deficiency of lysosomal glucocerebrosidase (see for review, Barranger & Ginns, 1989). Classically, the disease has been divided in three subtypes on the basis of clinical signs and symptoms. Type I, the least severe chronic non-neuronopathic form has a high incidence (carrier frequency 1:20) among the Ashkenasi Jewish population (Matoth et al., 1987). Type II has an acute, neuronopathic onset and type III is a subacute neuronopathic form of Gaucher disease. The Norrbottnian type of Gaucher disease prevailing in Sweden resembles type III (Dreborg et al., 1980). In all subtypes of Gaucher disease glucosylceramide is stored in the cells of the reticuloendothelial system.

In fibroblasts glucocerebrosidase is produced as a glycosylated 62.5 kDa precursor, which is processed via a 66 kDa intermediate to mature enzyme of 59 kDa. This processing is not proteolytic, but involves alterations of the carbohydrate chains, from high mannose to complex type (Aerts et al., 1986; Jonsson et al., 1987; Van Weely et al., 1990). Attempts have been made to discriminate between the different clinical phenotypes by immunological procedures, and by studying the physicokinetic parameters of the mutant enzymes (Ginns et al., 1982; Ginns et al., 1983; Beutler et al., 1984; Grabowski et al., 1985a; Beutler & Kuhl, 1986; Bergmann & Grabowski, 1989; Aerts et al., 1990). Furthermore, the posttranslational modification and intracellular localization of glucocerebrosidase were compared (Willemsen et al., 1987). Based on the results of these experiments it was predicted that multiple mutant alleles would exist and account for the clinical heterogeity in Gaucher disease (Grabowski et al., 1985a). Genetic heterogeneity was even demonstrated in the Ashkenasi Jewish population (Beutler et al., 1984).

The cDNA coding for glucocerebrosidase has been cloned (Ginns et al., 1984, 1985; Sorge et al., 1985a; Tsuji et al., 1986; Reiner et al., 1987). A PvuII polymorphism in the glucocerebrosidase gene was used to demonstrate that in the Ashkenasi Jewish population, several mutant alleles must have arisen independently (Sorge et al., 1985b).

Gross genetic abberrations were not observed, and mRNA analysis showed no quantitative and qualitative abnormalities (Graves et al., 1986; Ohashi & Eto, 1989). More detailed analysis of mutant cDNA's and genomic sequences revealed the existence of multiple allelic mutations, and Table 5 lists the mutations and their distribution among the different ethnic groups and clinical subtypes. Some correlations between genotype and phenotype seem to exist. The most prevalent mutation among Ashkenasi Jewish patients was identified as an adenine to guanidine transition (Asn^{370}→Ser) in exon IX of the glucocerebrosidase gene (Tsuji et al., 1988), and accounts for approximately 70% of the mutant alleles. Among patients it occurs in homozygous and heterozygous form (Tsuji et al., 1988; Theophilus et al., 1989a;

Zimran et al., 1990a). The same mutation has also been found in type 1 patients of other ethnic origins, but has uptill now not been discovered in type 2 and type 3 Gaucher disease. A linkage of this mutation with the above-mentioned PvuII polymorphism has been described (Zimran et al., 1990b).

A second common mutation in Gaucher disease is a T→C transition in exon X, changing leucine^{444} into proline. Screening for this mutation is feasible, because it creates a NciI site (Tsuji et al., 1987b). In a first series of patients this mutation was not encountered in homozygous form among 20 type 1 patients, but of 16 patients with type 2 or type 3 Gaucher disease, 9 appeared to be homozygous for the Leu^{444} → Pro mutation. These results were extended by others (Wigderson et al., 1989; Theophilus et al., 1989a; Zimran et al., 1989). The few type I patients that were homozygous for the Leu^{444} mutation had a more severe phenotype (Theophilus et al., 1989a).

Also the occurrence of point mutations in the isolated Norrbottnian type of Gaucher disease was investigated. Genealogic studies had indicated the existence of one, or possibly two the mutations within this population (Iselius et al., 1989). In a search for RFLP's a MspI polymorphism was found to segregate with the disease (Dahl et al., 1988). Recently it was discovered that this MspI-polymorphism actually represents the Leu^{444}→Pro mutation (Dahl et al., 1990a; 1990b). This indicates that the Leu to Pro mutation is associated with the milder subacute, rather than with the severe acute neuronopathic form of Gaucher disease.

With respect to the genotype-phenotype correlation it is tempting to speculate that the Asn^{370}→Ser mutation (exclusively found in type I Gaucher disease) does not effect glucocerebrosidase in such a way that neuropathological symptoms ensue. In line with this hypothesis is the observation that expression of the Asn^{370}→Ser mutated cDNA in insect cells results in the production of an enzyme with residual catalytic activity (Grace et al., 1990). It has been demonstrated that also in fibroblasts from patients with the Asn^{370}→Ser mutation catalytically active enzyme is present, albeit with altered kinetic properties (Grabowski et al., 1985b; Osiecki-Newman et al., 1987; Grace et al., 1990). In contrast, expression of Leu^{444} to Pro mutated cDNA in COS cells led to a completely inactive enzyme, stressing the severity of this latter mutation (Wigderson et al., 1989).

It seems illogical, that the Leu^{444}→Pro mutation occurs in both type II and type III disease in a homozygous form. One explanation might be the existence of additional mutations in the same allele causing the more severe type II phenotype. Indeed, a mutant allele was identified with a cluster of three mutations including T^{1448} →C (Leu^{444} →Pro), G^{1483} →C (Ala^{456} →Pro) and G^{1497} →C (silent) (Zimran et al., 1989, 1990a; Hong et al., 1990; Latham et al., 1990). But it was demonstrated that this allele results from a recombination between the normal glucocerebrosidase gene and the pseudogene (Zimran et al., 1990a). The "pseudo-pattern" allele has been identi-

Table 5. Glucocerebrosidase gene mutations and their effects

Clinical subtype		Genotype	Reference numbers	References
Gaucher type 1	Ashkenasi Jews	Ser370[a] / Ser370	(1,2,3)	*(1) Theophilus et al., 1989a; (2) Zimran et al., 1990; (3) Tsuji et al., 1988; (4) Graves et al., 1988; (5) Latham et al., 1990; (6) Theophilus et al., 1989b; (7) Hong et al., 1990; (8) Tsuji et al., 1987b; (9) Masuno et al., 1990; (10) Wigderson et al., 1989; (11) Zimran et al., 1990a; (12) Dahl et al., 1990a; 1990b*
		Ser370 / Gln120[b]	(1,4)	
		Ser370 / Pro444[c]	(1,2,3)	
		Ser370 / Leu394[d]	(5,6)	
		Ser370 / ?	(1,2,3)	
		? / ?	(1)	
	others	Ser370 / ?	(1,2,7)	
		Ser370 / Pro444	(1,2)	
		Ser370 / complex3[e]	(2,5,7)	
		Ser370 / His409[f]	(6)	
		Pro444 / His409	(6)	
		Pro444 / Pro444 *	(1,5,8,9)	
		Pro444 / ?	(1,8,10)	
		complex3 / ?	(7,11)	
		Cys463[g] / ?	(7)	
Gaucher type 2		Pro444 / complex3	(5,7)	
		Pro444 / Pro444 **	(8,10)	
		Pro444 / Arg415[h]	(1,8)	
		Pro444 / ?	(1,10)	
		? / ?	(1)	
Gaucher type 3	Norrbottnian	Pro444 / Pro444	(5,12)	
	others	Pro444 / Pro444	(8)	
		Pro444 /?	(1,8,10)	
		Pro444 / Val409[i]	(6)	
		Leu394 / complex4[j]	(5,6)	

* More severe phenotype;
** Possibly one or both alleles complex3

Notes to Table 5

	Mutation	cDNA position[1]	Genomic position[2]		Amino acid	Effect on mRNA	Effect on protein	Frequency	Reference number
a	A → G	1226	exon 9	(5841)	Asn^{370} → Ser	normal	decreased specific activity[3]	most common allele among Askenasi Jews	(3)
b	G → A	475	exon 5	(3060)	Arg^{120} → Gln	normal	catalytically inactive	rare	(4)
c	T → C	1448	exon 10	(6433)	Leu^{444} → Pro	normal		frequent, 100 % among Norrbottnian	(10)
d	G → T	1297	exon 9	(5912)	Val^{394} → Leu	normal		rare	(6)
e	T → C G → C G → C	1448 1483 1497	exon 10	(6433) (6468) (6482)	Leu^{444} → Pro Ala^{456} → Pro Val^{460} (silent)	normal	rapidly degraded	more than once	(5,7,11)
f	G → C	1342	exon 9	(5957)	Asp^{409} → His	normal		rare	(6)
g	C → T	1504	exon 10	(6490)	Arg^{463} → Cys	normal	catalytically inefficient	rare	(7)
h	C → G	1361	exon 9	(5976)	Pro^{415} → Arg	normal		rare	(10)
i	A → T	1343	exon 9	(5958)	Asp^{409} → Val	normal		rare	(6)
j	G → C T → C G → C G → C	1342 1448 1483 1497	exon 9 exon 10	(5957) (6433) (6468) (6482)	Asp^{409} → His Leu^{444} → Pro Ala^{456} → Pro Val^{460} (silent)	normal		rare	(5)

[1] From the first in-frame ATG-codon
[2] Horowitz et al., 1989
[3] Grace et al., 1990

fied in seven patients (Zimran et al., 1989, 1990a; Hong et al., 1990). The earlier identification of the Leu^{444} →Pro mutation by oligonucleotide hybridization, or by using the NciI-RFLP (Tsuji et al., 1987b; Theophilus et al., 1989a; Wigderson et al., 1989) may therefore represent Leu^{444} →Pro mutations as well as mutations caused by recombination. It has been suggested, that the pseudo-pattern allele is associated with a more severe expression of Gaucher disease than the Leu^{444} →Pro mutation alone (Latham et al., 1990). That a recombination between the pseudogene and the normal gene is not extremely rare, was demonstrated by the finding of a second "pseudo-pattern" allele. This allele had in addition to the three mutations described above, a G→C (Asp^{409} →His) transversion, derived from the pseudogene (Latham et al., 1990).

2.6 Mutations and lysosomal enzyme realization

Insertions/deletions

Insertions and deletions of different kind have been observed in lysosomal storage disorders. Among 130 cases of Fabry disease (α-galactosidase deficiency) six different gene rearrangements were discovered. In five cases it concerned a gene deletion, each time of varying length and at a different position in the α-galactosidase gene. In one case a partial gene duplication was encountered, involving exons 2-6. As a consequence, the α-galactosidase activity was completely deficient. (Bernstein et al., 1989). Investigation of the breakpoints in these cases identified short direct repeats that are involved in the gene rearrangements (Kornreich et al., 1990; Fukuhara et al., 1990). In the other X-linked lysosomal disease, iduronate 2-sulfate deficiency (Hunter disease) gene deletions were found in 7 of 20 patients. In two of these the whole gene seemed to be deleted (Wilson et al., 1990).

Sometimes an insertion in the mRNA can be found. The common 4 bp insertion in the HEX A gene (discussed above) leads to unstable mRNA (Myerowitz & Costigan, 1988; Paw & Neufeld, 1988). A point deletion of a cytidine residue (bp 1510) in the hexosaminidase α-chain causes a frameshift and premature chain termination. The mutant precursor is labile (Lau & Neufeld, 1989). A point mutation in intron 12 of the HEX B gene creates a new splice-acceptor site 24 bp in front of the usual site. This leads to an insertion of 24 base pairs in the mRNA and a slightly larger then normal, and labile ß-chain precursor (Nakano & Suzuki, 1989; Dlott et al., 1990).

A similar splice site mutation might be the cause of the insertion of 11 amino acids in the saposin B (SAP-1) protein of a SAP-1 deficient patient (Zhang et al., 1990). This insertion disturbs the hydrophobicity pattern of the protein, and probably results in a protein that is more amenable to degradation.

Glycosylation site mutations

Two mutations in glycosylation sites of lysosomal proteins have been described. The first concerns arylsulfatase A. The enzyme is glycosylated at two of the three potential sites. One of the A→G transitions found in the "pseudo deficiency" allele changes Arg^{350} →Ser, which leads to the loss of a glycosylation site. But, mutation analysis did not reveal any effect on the activity and stability of arylsulfatase A, despite the loss of a carbohydrate chain (Gieselmann et al., 1989).

Another example of a glycosylation site mutant concerns a C to T transition in codon 23 of saposin B (SAP-1), changing a threonine into an isoleucine residue. In this way, the only glycosylation site in the protein is abolished, and this could be the reason for its rapid degradation in vivo (Kretz et al., 1990; Rafi et al., 1990). From the two examples cited here, it can be concluded that the abolition of a glycosylation site may lead to a protein deficiency, but may also go unnoticed.

Mutations and protein domains

Mutations may elucidate some of the functions of protein domains. For instance, several mutations in exon 13 (Table 4) of the HEX A-gene affect the intracellular transport or the association with the ß-subunit (Nakano et al., 1988; Lau & Neufeld, 1989; Paw et al., 1990a). From these observations it may be inferred that sequences in exon 13 (coding for the carboxyterminal part of the α-chain) are important for the proper folding of the hexosaminidase α-chain. Another example is the B_1-variant of G_{M2}-gangliosidosis, which lacks activity towards certain substrates. In 5 out of 6 patients from all over the world an Arg^{178} to His substitution (Table 4) (Ohno & Suzuki, 1988c; Tanaka et al., 1988) was demonstrated in the hexosaminidase α-chain. More recently, an Arg^{178}→Cys mutation was found in another patient with the B_1-variant (Tanaka et al., 1990a), stressing the importance of Arg^{178} for proper catalytic activity. When the Arg^{178}→His mutation was introduced at the corresponding position of the homologous hexosaminidase ß-chain, loss of catalytic activity was noted (Brown et al., 1989).

2.7 Diagnostic and clinical applications

Carrier detection and prenatal diagnosis

Since there is no effective treatment for lysosomal storage diseases, prevention is of utmost importance. This can be achieved by genetic counseling of couples at risk, followed by carrier detection and/or prenatal diagnosis. Carrier detection by population screening is only feasible when the disease occurs in a high frequency, and when a simple, reliable and cheap test is available. A screening program for carrier detection of Tay-Sachs disease based on reduced enzyme levels has been effective for more than a decade among the Ashkenasi Jewish population (see for review,

Sandhoff et al., 1989). In a recent study, the results of carrier screening at the DNA level and the enzyme activity level were compared (Triggs-Raine et al., 1990). Only in one case a discrepancy was found. A woman was diagnosed as a carrier by the DNA test, whereas she seemed to be a homozygote normal using the enzyme activity test. The difference was explained by the fact that the woman was pregnant at the time of testing. It is known that pregnancy enhances the hexosaminidase A-like activity in the serum. The study indicates that carrier testing at the DNA level might sometimes be advantageous (Triggs-Raine et al., 1990). However, screening at the enzyme activity level remains the method of choice, because it allows the detection of yet unidentified mutant alleles (Paw et al., 1990b).

The presence of a wide variety of mutations in all other lysosomal storage diseases precludes carrier detection at the DNA level. The only exception might be the Norrbottnian type of Gaucher disease, because only one mutation seems to be responsible for its occurrence in the Swedish population (Dahl et al., 1990a; 1990b).

Prenatal diagnosis for lysosomal enzyme deficiencies is routinely performed by measuring the enzyme activity in chorionic villi or amniotic fluid cells (Kleijer 1986; 1990). In rare cases, when it is difficult to discriminate between a carrier and a patient, the analysis of DNA might be helpful. In case the mutation is not known, the use of intragenic polymorphisms may be considered. Such polymorphisms have been described for acid α-glucosidase, glucocerebrosidase, and arylsulfatase A (Hoefsloot et al., 1990a; 1990b; Tzall et al., 1990a; 1990b; Sorge et al., 1985b; Polten and Gieselmann, 1990; Herzog et al., 1990).

Therapy

There are two ways to envisage the use of cloned lysosomal genes for the treatment of lysosomal storage disorders. One is, to overexpress the cloned genes in eukaryotic cells for the production of large amounts of enzyme for use in enzyme replacement therapy. Although enzyme replacement therapy has been little effective in the past, more recent reports are encouraging. For instance, significant improvement of bone deformations, increased platelet count, and decreased phagocytic activity in the spleen, were reported in a patient with Gaucher disease type I after treatment with modified glucocerebrosidase isolated from human placenta (Barton et al., 1990). Also studies by Van der Ploeg et al. (1991) point to the feasibility of receptor mediated enzyme replacement therapy. The uptake of intravenously administered mannose 6-phosphate containing acid α-glucosidase in mouse skeletal muscle and heart suggests that the application of this therapy in adult glycogenosis type II might be beneficial (Van der Ploeg et al., 1991). The applicability of enzyme replacement therapy in lysosomal storage diseases with neurological involvement is questionable, because of the blood-brain barrier.

Another way to use the coding sequences of lysosomal enzymes for treatment of

patients might be gene therapy. Most attention is presently focused on the correction of bone marrow stem cells of the patient with a retroviral vector carrying the gene of interest (Sorge et al., 1987; Correll et al., 1989; Nolta et al., 1990; Wolfe et al., 1990). When retransplanted, the progeny of these cells is expected to replace the affected blood cells and tissue macrophages. In addition, correction of other cell types may occur through uptake of secreted enzyme. Apart from the fundamental problems related to the infection of early bone marrow stem cells and their maintenance in the bone marrow (Valerio et al., 1989; Van Beusechem et al., 1990), some specific problems have to be overcome before this technique can be applied successfully for the treatment of lysosomal storage disorders. For instance, overexpression of the implanted gene will be required, since clinical trials over the last six years have shown that transplantation with normal bone marrow cells is not sufficient (see for review, Van der Ploeg, 1989).

2.8 References

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CHAPTER 3

The experimental work: introduction and discussion

3.1 Glycogenosis type II

Generalized glycogenosis (glycogenosis type II, Pompe disease) was described for the first time in 1932 by the Dutch physician J.C. Pompe (Pompe, 1932). Three decades later acid α-glucosidase (acid maltase) deficiency was discovered to be the primary defect in this disorder (Hers, 1963). At the same time, the lysosomal localization of acid maltase was established (Lejeune et al., 1963), which made Pompe disease the first and classical example of a lysosomal storage disorder.

Patients with glycogenosis type II (GDS II) are usually classified as infantile or late onset variants, depending on the age of onset and the severity of the clinical symptoms (see for review, Hers et al., 1989). In the most severe, infantile form of glycogenosis type II pathological signs are manifest at birth. On presentation the infants have poor motor development, dyspnea and failure to thrive. Other characteristic features are hepatosplenomegaly, severe hypotonia, and cardiac enlargement with an altered electro-cardiogram. Excessive storage of glycogen is found in many tissues. Death follows usually within the first year of life, due to cardiorespiratory failure, pneumonia, or aspiration.

The juvenile form of glycogenosis type II has an onset in the first decade of life, whereas symptoms can be delayed till the third or fourth decade in late-onset variants. In these latter clinical subtypes there is no cardiac involvement, and mainly the skeletal muscles of limb and girdle are affected. Death usually results from respiratory failure. There is no effective treatment for the disease and clinical management is restricted to alleviation of symptoms. Glycogenosis type II is inherited as an autosomal recessive trait, like most other lysosomal storage disorders. The incidence is approximately 1 in 100,000. The large heterogeneity in clinical presentation has been attributed to varying levels of residual acid α-glucosidase activity among the patients. The acid α-glucosidase deficiency is virtually complete in infantile patients, whereas adult patients may have as much as 25% of the normal activity (Mehler & DiMauro, 1977; Shanske et al., 1986; Reuser et al., 1978, 1982, 1987; Van der Ploeg et al., 1987, 1988, 1989; Hoefsloot et al., 1990a). The observed correlation is rather strict, but few exceptions have been reported in the form of adult patients with unusually low enzyme activities (Beratis et al., 1983; Reuser et al., 1987).

3.2 Acid α-glucosidase

Lysosomal or acid α-glucosidase (E.C. 3.2.1.3) is an exoglycosidase (gamma-

amylase) with an acidic pH optimum. The enzyme degrades glycogen to its monosaccharide building blocks (glucose) and, therefore, has α-1,4 and α-1,6 glucohydrolase activity. Besides for the natural substrates glycogen, isomaltose and maltose, the enzyme exhibits activity towards some artificial oligoglucosides (Jeffrey et al., 1970a, 1970b; Palmer, 1971a, 1971b; Rosenfeld, 1975; Koster & Slee, 1977). The catalytic activity is often assayed with 4-methyl-umbelliferyl-α-D-glucopyranoside. B y in vitro translation of RNA extracted from human hepatoma cells the molecular mass of the unglycosylated acid α-glucosidase precursor was estimated to be 100 kD. The addition of sugar chains after translocation into microsomes increases the apparent molecular mass with approximately 12 kD (Van der Horst et al., 1987). The first form of acid α-glucosidase detectable in pulse-chase studies with cultured human fibroblasts is a precursor of 110 kD (Hasilik & Neufeld, 1980a, 1980b; Reuser et al., 1985, 1987). Some of the carbohydrate chains are phosphorylated in the cis-Golgi compartment, and obtain the mannose 6-phosphate recognition marker (Hasilik & Neufeld, 1980a, 1980b; Reuser et al., 1985). The acid α-glucosidase precursor undergoes several proteolytic changes during intracellular transport and upon arrival in the lysosomes, whereby the molecular mass is stepwise reduced till 76 and 70 kD. A relatively stable processing intermediate has a molecular mass of 95 kD. The intracellular compartments in which proteolytic processing occurs are not known, but the 95 kD, the 76 kD, and the 70 kD forms of acid α-glucosidase probably arise after the precursor has passed the Golgi complex (Oude Elferink et al., 1985; H.A. Wisselaar et al., unpublished results). In human urine, a 110 kD precursor form is found (Oude Elferink et al., 1984; 1989).

3.3 Acid α-glucosidase deficiencies

When acid α-glucosidase deficiencies were studied in fibroblasts from patients with different forms of glycogenosis type II, defects were found at all stages of enzyme realization. No gross deletions were observed in the DNA, but complete deficiency, partial deficiency, as well as normal mRNA levels were encountered (Hoefsloot et al., 1988; Reuser et al., 1988; Martiniuk et al., 1990. See also Chapter 9). In some cases synthesis of the acid α-glucosidase precursor was not detectable by in vitro labelling procedures (Van der Ploeg et al., 1989). In others, the precursor appeared to be synthesized normally, but no processing to mature enzyme was observed. Two of these latter mutants showed defective phosphorylation (Reuser et al., 1985). Most adult variants were characterized by a reduced rate of enzyme synthesis, resulting in a lower than normal amount of mature acid α-glucosidase in the lysosomes. In one particular case aberrant proteolytic processing was observed (Reuser et al., 1987). Besides these mutants affected in biosynthesis and/or intracellular transport of acid α-glucosidase, healthy individuals were described with a

polymorphism causing the enzyme to have a reduced affinity for glycogen (Swallow et al., 1975; 1989; Suzuki et al., 1988). At first sight there does not seem to be a correlation between the nature of the molecular defect and the clinical phenotype. However, in conjunction with other observations it appears significant that a complete lack of precursor synthesis was not detected in any of the adult, in contrast to the infantile patients. Moreover, immunocytochemical studies have demonstrated that there is more acid α-glucosidase in the lysosomes of patients with late-onset than with early-onset forms of the disease (see Van der Ploeg, 1989 for review). The lysosomal localization of the enzyme is a prerequisite for the generation of catalytic activity. As discussed in paragraph 3.1, the level of this activity appears to be the primary determinant of the clinical phenotype.

3.4 The sequence of acid α-glucosidase

Characteristic structural and functional features of a protein can be deduced from the primary amino acid sequence. For instance, the presence of semi-conserved signal peptides provides information on the intracellular routeing (see Chapter 1), and the occurrence of the Asn-X-Ser/Thr tripeptide marks the positions of potential glycosylation sites. Sequence similarities with other proteins help to delineate functionally important domains, and can be informative concerning the evolutionary origin of proteins (see Chapter 2). It is evident, that the primary structure of a protein must be known before the mutations in patients can be identified, and the effect of these mutations can be assessed. The experimental work in this thesis involves the isolation and the characterization of the cDNA and the gene coding for acid α-glucosidase. It deals with structural features of acid α-glucosidase, and aspects of post-translational modification and intracellular transport. The knowledge is applied in studies on the cause of clinical heterogeneity in glycogenosis type II.

Structural aspects

The isolation of cDNA clones coding for acid α-glucosidase is described in detail in Chapter 4 (Hoefsloot et al., 1988). The first cDNA clones were obtained by antibody screening of a λgt11 expression library and were identified in several ways. When used as a probe on Southern blots of human-rodent hybrid cell lines they were found to hybridize specifically to only one particular, small region of human chromosome 17, known to contain the acid α-glucosidase gene. On Northern blots the cloned cDNA recognized a messenger of approximately 3.8 kb in RNA isolated from cells of a patient with a normal acid α-glucosidase production, but this messenger RNA was absent in cells from a patient without enzyme production. The identity of the clones was established unambiguously by sequence analysis. The predicted amino acid sequence of the encoded protein was found to match exactly the sequence of

several tryptic and CNBr peptides of acid α-glucosidase. When the different cDNA clones were lined up they comprised 3624 base pairs. A consensus sequence for translation-initiation (Kozak, 1987) situated around the first in-frame ATG codon indicated the position of the N-terminal methionine residue. Moreover, a stretch of amino acid residues with typical characteristics of a signal peptide followed this methionine residue. Given the position of the first stop codon, the acid α-glucosidase precursor is predicted to consist of 952 amino acids.

Since the N-terminal amino acid sequence of the 110 kD precursor and the 76 kD and 70 kD mature forms of acid α-glucosidase had been determined, the sites of proteolytic processing became immediately evident. Interestingly, the N-terminus of the 110 kD precursor (isolated from human urine) appeared to be located 70 amino acid residues after the first methionine. Thus, it was concluded that proteolytic processing occurs before the precursor is excreted in the urine. The 69 amino acid long propeptide may either be removed as one piece, or the signal peptide may be cleaved off first at the most probable site between amino acid residue 24 and 25 (Barnes & Wynn, 1988). The overall complexity of the proteolytic processing became even more evident from the position of the N-termini of the 76 and 70 kD polypeptides. The 76 kD mature enzyme appeared to be derived from the 110 kD precursor by both N- and C-terminal trimming. The further maturation from 76 kD to 70 kD seemed to be exclusively an N-terminal event.

The amino acid sequence indicated further the position of seven potential glycosylation sites (Asn-X-Ser/Thr). Four of these sites are located within the predicted boundaries of the 70 kD species. The 76 kD form of acid α-glucosidase has one additional site. Sites 6 and 7 are located at the C-terminal end of the protein and are lost during maturation of the 110 kD precursor. Mutsaers et al. (1987) had shown previously that (mature) human placental acid α-glucosidase accomodates four to five carbohydrate chains. This then would mean, that all potential glycosylation sites in the mature enzyme are being used. More recently, it was demonstrated by in vitro mutagenesis that all seven sites of the 110 kD precursor are glycosylated (M.M.P. Hermans et al., unpublished results).

The amino acid sequence revealed another most interesting aspect. Acid α-glucosidase resembled two other enzymes with a similar substrate specificity for maltose, i.e. sucrase and isomaltase. These latter disaccharidases are encoded by a single gene. Given the degree of mutual homology of acid α-glucosidase, sucrase and isomaltase, this gene must have arisen by gene duplication from the same ancestral gene as acid α-glucosidase. A model describing the evolutionary origin of the three enzymes is depicted in Figure 1. Evolutionary changes in the primary sequences have apparently led to the distinct substrate specificities and physical properties of the three enzymes. One of the most obvious differences is the intracellular localization and the posttranslational modification of sucrase and isomaltase compared with

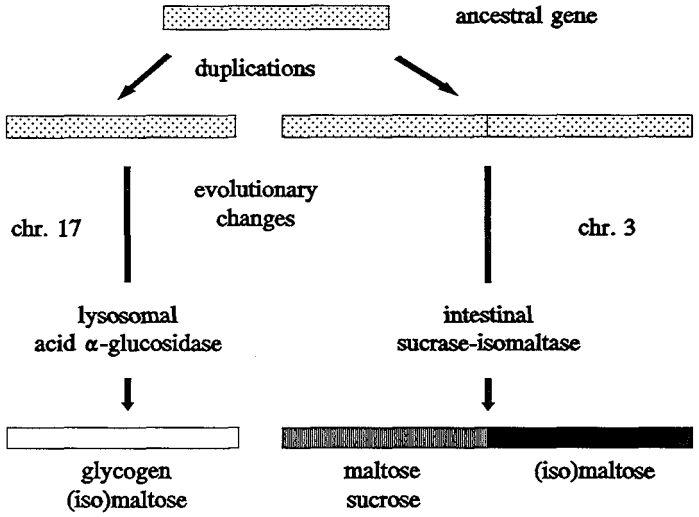

Figure 1

lysosomal α-glucosidase. The common precursor of sucrase and isomaltase has a signal peptide that is not cleaved off and anchors isomaltase in the apical membrane of intestinal epithelial cells. In the lumen of the intestine the precursor is cleaved by pancreatic enzymes, forming the sucrase and isomaltase subunits. More extensive proteolytic processing does not occur, and sucrase remains non-covalently linked to isomaltase (see for review, Semenza & Auricchio, 1989). The build-in signals for differential transport and function of acid α-glucosidase and sucrase-isomaltase remain to be discovered. So far, the sequence homology has helped to delineate the active site domain of acid α-glucosidase. Conduritol ß-epoxide had been used to label the essential carboxylates in the catalytic sites of sucrase and isomaltase (Quaroni & Semenza, 1976; Hunziker et al., 1986). The surrounding amino acid sequence appeared to be extremely conserved in acid α-glucosidase. This way the catalytic residue was assigned tentatively.

Also the gene coding for acid α-glucosidase was analyzed. The results are discussed in detail in Chapter 6 (Hoefsloot et al., 1990c). Overlapping phage λ clones containing together the entire gene were isolated from a genomic DNA library. The exon-intron boundaries were established, and the gene was found to consist of 20 exons. Notably, a small intron of 101 base pairs was located in the middle of the predicted acitve site domain. A similarly positioned intron was not present in the human and rabbit genomic sequences encoding isomaltase. The first intron in the acid α-glucosidase gene was located in the 5' untranslated region of the cDNA. Further analysis of this region by primer extension showed that approximately 200 base pairs

were missing at the 5' untranslated region of the cloned cDNA. The promoter activity of the sequences upstream of the transcription-initiation site was tested using the CAT-assay, and the minimal boundaries of the promoter region were established. Acid α-glucosidase appeared to have a typical GC-rich house keeping gene promoter, without apparent TATA or CCAAT consensus sequences. The presence of a potential AP2-binding site may be of significance for regulation of gene expression.

Functional aspects

To confirm the integrity of the isolated acid α-glucosidase cDNA constructs were made containing the full length coding sequence. In Chapter 5 (Hoefsloot et al., 1990b) the expression of the constructs in transiently transfected COS cells is described. The cDNA-derived enzyme was found to have the same characteristics as acid α-glucosidase from normal human fibroblasts when the processing and the catalytic activity were compared. Due to the high level of expression, the enzyme could easily be visualized in the compartments of the biosynthetic pathway and in the lysosomes, using imunocytochemistry. Unlike fibroblasts, transfected COS cells secreted a fair amount of 110 kDa precursor. When added to the culture medium of enzyme deficient fibroblasts, the precursor was endocytosed via the mannose 6-phosphate receptor, transported to the lysosomes, and converted to mature enzyme. As a result, the glycogen storage disappeared. Thus, the integrity of cloned acid α-glucosidase cDNA was proven.

Besides at the usual intracellular locations, acid α-glucosidase was also detected at the plasma membrane of transfected COS cells. The mode of attachment was investigated to some extent. Non-specific binding and binding to the mannose 6-phosphate receptor were excluded. The only domain in acid α-glucosidase with high enough hydrophobicity to cause direct association with the membrane is the signal peptide. Considering the homology between acid α-glucosidase and the membrane-anchored sucrase-isomaltase complex, attachment via an uncleaved signal peptide is still one of the possibilities. It is less likely that the membrane association is an artifact of the transfection procedure. Membrane association of the acid α-glucosidase precursor was established biochemically, and the precursor was demonstrated to be present also on the apical membrane of intestinal epithelial cells, together with sucrase-isomaltase (Fransen et al., 1989). It can be envisaged that the three enzymes have some sorting signals in common.

3.5 Mutations, patients and polymorphisms

Besides the normal cDNA, a mutant acid α-glucosidase cDNA was discovered among the isolated clones. The clone differed in only one base pair, causing a change from a tryptophan into an arginine at position 402 of the amino acid sequence.

Expression of this mutant cDNA in COS cells revealed that the mutation had a deleterious effect on both the proteolytic processing and the catalytic activity of acid α-glucosidase. Immunocytochemistry showed that the intracellular transport of the mutant protein was blocked at the level of the Golgi complex (Chapter 5). Further transport is possibly prevented by a quality-control system at the Golgi-exit, as described in Chapter 1.

A combination of techniques was used to study the inheritance of mutant acid α-glucosidase alleles in an unique family with glycogenosis type II (Chapter 7; Hoefsloot et al., 1990a). The occurrence of both the adult and the infantile form of glycogenosis type II in the first and the third generation, respectively, provided an excellent opportunity to investigate the relation between genotype and phenotype. Man-mouse somatic cell hybrids were created to segregate the two human chromosomes 17, carrying the acid α-glucosidase alleles of the adult patient. The chromosomes were identified by restriction fragment length polymorphisms, and the acid α-glucosidase species produced by each of the alleles were analyzed separately. In this way, the adult case of GSDII was shown to be a genetic compound with one allele accounting for 30 % residual acid α-glucosidase activity, and one silent allele. The latter allele was shown to be inherited by the grandchild, who obtained another infantile type GSD II allele from its mother. It was concluded that the clinical phenotype in this family is exclusively determined by the level of residual acid α-glucosidase activity.

A further investigation of heterogeneity was performed in South-African cases of GSD II (Chapter 8; Van der Ploeg et al., 1989). Two rare mutations were discovered, the first being the total absence of acid α-glucosidase protein and mRNA in two unrelated black baby girls. The second concerned a smaller than normal acid α-glucosidase precursor and lack of proteolytic processing in two sibs of a consanguineous Indian family with infantile GSD II. The acid α-glucosidase mRNA seemed normal in size and amount. In this family, the mutant allele was characterized by a 0.5 kb insertion, but a direct relation between the insertion and acid α-glucosidase deficiency was excluded. Although the insertion was not observed in acid α-glucosidase alleles of 18 healthy Caucasian individuals, it appeared to occur rather frequently in the Asian population, as described in Chapter 9. Therefore it represents a polymorphism. Other polymorphisms in the acid α-glucosidase gene and mutant alleles were discovered in the Caucasian as well as in the Asian population, and their frequencies were established (Hoefsloot et al., 1990d, 1991; Tzall et al., 1990a, 1990b). In particular cases, these polymorphisms may be used to discriminate between glycogenosis type II carriers and patients.

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CHAPTER 4

Primary structure and processing of lysosomal α-glucosidase; homology with the intestinal sucrase-isomaltase complex[1]

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Summary

Lysosomal α-glucosidase (acid maltase) is essential for degradation of glycogen in lysosomes. Enzyme deficiency results in glycogenosis type II. The amino acid sequence of the entire enzyme was derived from the nucleotide sequence of cloned cDNA. The cDNA comprises 3639 nt, and hybridizes with a messenger RNA of ~ 3.6 kb, which is absent in fibroblasts of two patients with glycogenosis type II. The encoded protein has a molecular mass of 105.3 kD and starts with a signal peptide. Sites of proteolytic processing are established by identification of N-terminal amino acid sequences of the 110 kD precursor, and the 76 kD and 70 kD mature forms of the enzyme encoded by the cDNA. Interestingly, both amino-terminal and carboxy-terminal processing occurs. Sites of sugar-chain attachment are proposed. A remarkable homology is observed between this soluble lysosomal α-glucosidase and the membrane-bound intestinal brush border sucrase-isomaltase enzyme complex. It is proposed that these enzymes are derived from the same ancestral gene. Around the putative active site of sucrase and isomaltase, 10 out of 13 amino acids are identical to the corresponding amino acids of lysosomal α-glucosidase. This strongly suggests that the aspartic acid residue at this position is essential for catalytic function of lysosomal α-glucosidase.

Introduction

Lysosomal α-glucosidase (acid α-glucosidase, acid maltase, EC 3.2.1.3) is a γ-amylase with specificity for glycogen and several natural and synthetic oligoglucosides. The enzyme is most active at low pH (4.0-5.0), and hydrolyzes both α-1,4 and α-1,6 glycosidic linkages (Jeffrey et al., 1970a, 1970b; Palmer, 1971a, 1971b;

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Rosenfeld, 1975; Koster and Slee, 1977). Deficiency of acid α-glucosidase in mammals and birds (for review, see Walvoort, 1983) leads to accumulation of glycogen in the lysosomes and results in glycogenosis type II (GSD II or Pompe disease), an autosomal recessive disease (Hers, 1963). The acid α-glucosidase gene has been mapped to chromosome 17, q21-23 (Solomon et al., 1979; Martiniuk et al., 1985).
The clinical presentation of glycogenosis type II in humans varies considerably, which has led to classification of subtypes. Infantile glycogenosis type II is a rapidly progressive disorder with onset of symptoms shortly after birth. Cardiorespiratory insufficiency is the major cause of death in the first or second year of life. In milder late-onset forms of the disease, the heart is not affected and the main symptom is skeletal muscle weakness (Howell and Williamson, 1983). Clinical heterogeneity originates primarily from different mutations of acid α-glucosidase, but secondary genetic and epigenetic factors may be involved (Beratis et al., 1983; Reuser et al., 1985, 1987).

In the course of studying the molecular basis of clinical heterogeneity, information was obtained on the biosynthesis of normal and mutant acid α-glucosidase. *In vitro* translation of acid α-glucosidase mRNA showed an apparent molecular weight of 100 kD for the unglycosylated enzyme precursor. The precursor enters the lumen of the endoplasmic reticulum cotranslationally and is glycosylated. This results in a 110 kD molecular species (Van der Horst et al., 1987). The biosynthesis of acid α-glucosidase in cultured fibroblasts proceeds with transfer of the enzyme to the Golgi complex where high-mannose type oligosaccharide side-chains are phosphorylated (Hasilik and Neufeld, 1980a, 1980b; Reuser et al., 1985). Subsequent binding of the precursor to the mannose-6-phosphate receptor ensures further transport to the lysosomes, where mature acid α-glucosidase species of 76 and 70 kD are most abundant. Maturation is a result of proteolytic processing, but the sites of processing and the mechanism are unknown. Acid α-glucosidase species of 105, 100 and 95 kD have been identified as processing intermediates (Reuser et al., 1985; Oude Elferink et al., 1985).

Studies on the biosynthesis of lysosomal α-glucosidase in fibroblasts from patients have led to the conclusion that different mutations are underlying functional enzyme deficiency. Among these are mutations with a specific effect on enzyme synthesis, phosphorylation, proteolytic processing, catalytic activity, and intracellular localization of acid α-glucosidase (Reuser et al., 1985, 1987).

Cloning of the cDNA coding for acid α-glucosidase was undertaken to obtain information on the primary structure of the enzyme, to study the maturation process, and to investigate in a later stage the exact nature of the mutation in a number of mutant phenotypes. Recently the isolation of a partial cDNA coding for acid α-glucosidase was described (Martiniuk et al., 1986). We now report the full-length coding sequence for acid α-glucosidase. Sites of amino-terminal processing are

established, and the attachment site of two sugar chains is predicted. Homology is shown between human lysosomal α-glucosidase, the rabbit intestinal sucrase-isomaltase complex and the partially cloned human isomaltase. Implications for the evolutionary origin of these proteins are discussed.

Results

Isolation of cDNA clones

A λgt11 human testis cDNA library was screened with a polyclonal antibody preparation against human placenta acid α-glucosidase, and nine clones remained positive after the rescreen. The inserts varied in length from 1.3 to 2 kb. Five of the

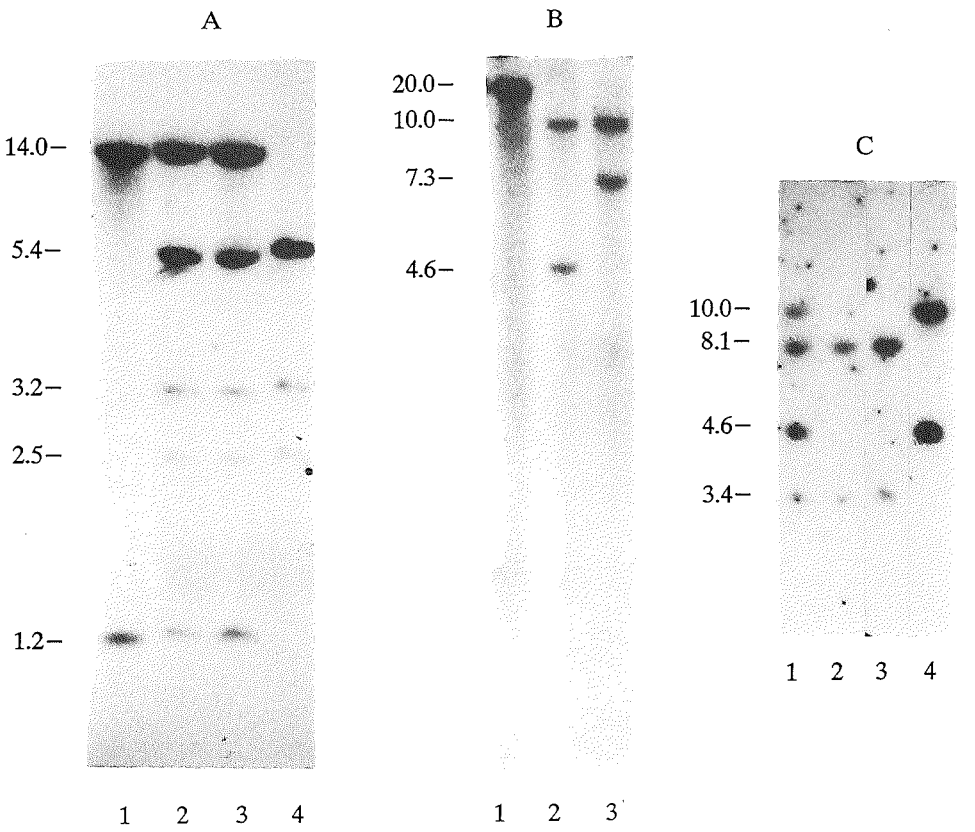

Figure 1. Southern blot probed with 2.0 kb acid α-glucosidase cDNA. Panel A: BamHI digested human placenta DNA (lane 1), 17CB18A DNA (lane 2), 17CB26B DNA (lane 3) and Chinese hamster (A23) DNA (lane 4). Panel B: Human placenta DNA digested with EcoRI (lane 1), HindIII (lane 2), and BglII (lane 3). Panel C: HindIII digested 60C2HAT DNA (lane 1), 60C2BUdR DNA (lane 2), mouse LTK$^-$ DNA (lane 2), and human placenta DNA (lane 4). Fragment lengths are indicated in kilobases.

nine inserts cross-hybridized. The longest of these (2.0 kb) was used as a probe on a Southern blot to test its hybridization with DNA from human chromosome 17 which carries the acid α-glucosidase locus. For this purpose, DNA of hamster-human somatic cell hybrids with no other human chromosomes than 17 was digested with *Bam*HI (Figure 1A). The 2.0 kb insert hybridized with 14 and 1.25 kb *Bam*HI fragments of placenta DNA (lane 1), which fragments were also present in the DNA of the two hybrid cell lines (lanes 2 and 3), but not in DNA of the Chinese hamster cell line A23 (lane 4). Instead, the probe hybridized with 5.4, 3.2, and 2.5 kb *Bam*HI fragments of Chinese hamster DNA. These same fragments were present in the DNA of the two hybrids, but not in placenta DNA.

Figure 1B shows that the 2.0 kb probe hybridized with a 20 kb fragment of *Eco*RI

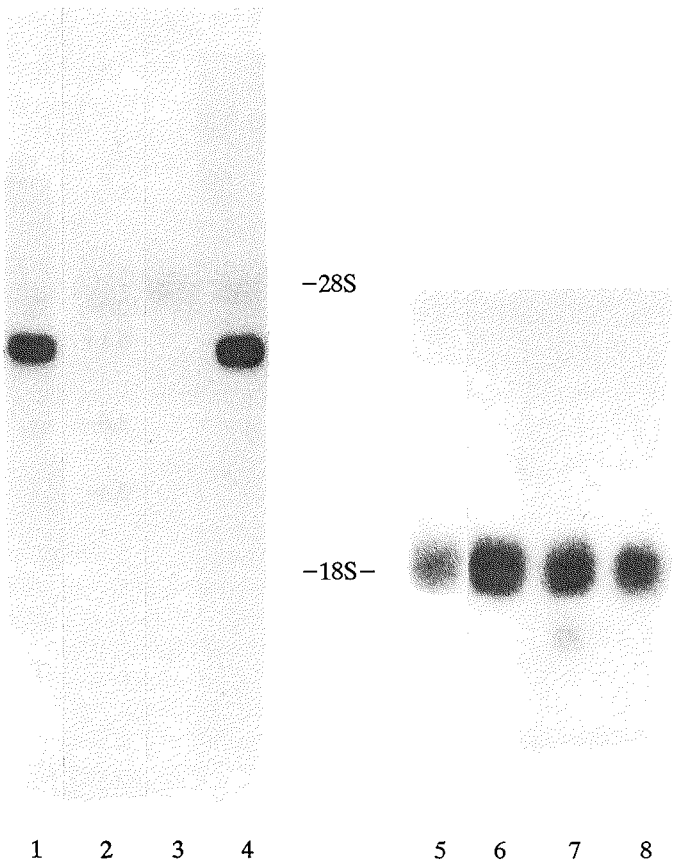

Figure 2. Northern blot of total RNA extracted from control fibroblasts (lane 1), and RNA extracted from fibroblasts of patients with infantile glycogenosis type II (lanes 2-4). The same blot was hybridized with an actin probe (lanes 5-8). The position of the ribosomal RNA bands of 28S and 18S is indicated.

digested placenta DNA (lane 1). Hybridization with *Hin*dIII digested DNA revealed 10- and 4.6 kb fragments (lane 2), and fragments of 10- and 7.3 kb were recognized when placenta DNA was digested with *Bgl*II (lane 3).

For further identification the 2.0 kb fragment was used as a probe on two different human-mouse hybrid cell lines (Figure 1C). One of these, 60C2HAT, contains only a small fragment of human chromosome 17 and has human acid α-glucosidase activity. The other, 60C2BUdR, is derived from 60C2HAT by selecting against this chromosomal fragment, and has lost activity for human acid α-glucosidase. In DNA of 60C2HAT (lane 1) the probe hybridized with the human 10 kb and 4.6 kb *Hin*dIII fragments that are also present in placenta DNA (lane 4). However, in DNA extracted from 60C2BUdR (lane 2), no human derived fragments could be observed to hybridize with the 2.0 kb probe, although the mouse fragments, present in LTK^- (lane 3) and 60C2HAT were detected.

Northern blot analysis of total RNA extracted from control fibroblasts showed a single hybridizing species of 3.6 kb (Figure 2, lane 1), which is long enough to code for a protein of 100 kD. In the same experiment RNA extracted from fibroblasts of three infantile glycogenosis type II patients was analyzed. No acid α-glucosidase was synthesized in fibroblasts of two of these patients as judged by immunoblotting (data not shown). In these two cases, no hybridizing mRNA was detected on the Northern blot, not even after a 3-fold increase in longer exposure (lanes 2 and 3). In cells of the third patient with a normal synthesis of inactive acid α-glucosidase, a normal amount of acid α-glucosidase mRNA was detected (lane 4). Similar amounts of RNA were applied in each lane, as judged from the signal obtained after hybridization of the same blot with an actin probe (lanes 5 to 8). No gene deletions were discovered by Southern blot analysis of DNA from these patients (data not shown). It is concluded from the analysis of cell hybrids and the Northern blot that the 2.0 kb insert represents part of the cDNA for human acid α-glucosidase.

Nucleotide and deduced amino acid sequences

In order to obtain longer clones, the original clone was used to screen a human placenta λgt11 cDNA library. Several positive clones were obtained and mapped using different restriction enzymes. Five overlapping clones appeared to have the same 3' end, three of these extended beyond the 5' end of the 2.0 kb-clone. The longest clone was 3.3 kb. One overlapping clone of 2.9 kb missed the 3' end but extended an extra 300 bp to the 5' end. Fragments of these clones were subcloned for sequencing purposes. The resulting nucleotide sequence of the combined clones is illustrated in Figure 3.

The cDNA is 3639 nt long, and the longest open reading frame with a length of 2880 nt starts at position 196 and ends with a stop codon at position 3076. In the open reading frame the first ATG is at position 220, therefore, the cDNA codes for a

```
                                                            60
CAGTTGGGAAAGCTGAGGTTGTCGCCGGGGCCGCGGGTGGAGGTCGGGGATGAGGCAGCA
                                                           120
GGTAGGACAGTGACCTCGGTGACGCGAAGGACCCCGGCCACCTCTAGGTTCTCCTCGTCC
                                                           180
GCCCGTTGTTCAGCGAGGGAGGCTCTGGGCCTGCCGCAGCTGACGGGGAAACTGAGGCAC
                                                           240
GGAGCGGGCCTGTAGGAGCTGTCCAGGCCATCTCCAACCATGGGAGTGAGGCACCCGCCC
                                                           300
TGCTCCCACCGGCTCCTGGCCGTCTGCGCCCTCGTGTCCTTGGCAACCGCTGCACTCCTG
                                                           360
GGGCACATCCTACTCCATGATTTCCTGCTGGTTCCCCGAGAGCTGAGTGGCTCCTCCCCA
                                                           420
GTCCTGGAGGAGACTCACCCAGCTCACCAGCAGGGAGCCAGCAGACCAGGGCCCCGGGAT
                                                           480
GCCCAGGCACACCCCGGCCGTCCCAGAGCAGTGCCCACACAGTGCGACGTCCCCCCCAAC
                                                           540
AGCCGCTTCGATTGCGCCCCTGACAAGGCCATCACCCAGGAACAGTGCGAGGCCCGCGGC
                                                           600
TGCTGCTACATCCCTGCAAAGCAGGGGCTGCAGGGAGCCCAGATGGGGCAGCCCTGGTGC
                                                           660
TTCTTCCCACCCAGCTACCCCAGCTACAAGCTGGAGAACCTGAGCTCCTCTGAAATGGGC
                                                           720
TACACGGCCACCCTGACCCGTACCACCCCCACCTTCTTCCCCAAGGACATCCTGACCCTG
                                                           780
CGGCTGGACGTGATGATGGAGACTGAGAACCGCCTCCACTTCACGATCAAAGATCCAGCT
                                                           840
AACAGGCGCTACGAGGTGCCCTTGGAGACCCCGCGTGTCCACAGCCGGGCACCGTCCCCA
                                                           900
CTCTACAGCGTGGAGTTCTCCGAGGAGCCCTTCGGGGTGATCGTGCACCGGCAGCTGGAC
                                                           960
GGCCGCGTGCTGCTGAACACGACGGTGGCGCCCCTGTTCTTTGCGGACCAGTTCCTTCAG
                                                          1020
CTGTCCACCTCGCTGCCCTCGCAGTATATCACAGGCCTCGCCGAGCACCTCAGTCCCCTG
                                                          1080
ATGCTCAGCACCAGCTGGACCAGGATCACCCTGTGGAACCGGGACCTTGCGCCCACGCCC
                                                          1140
GGTGCGAACCTCTACGGGTCTCACCCTTTCTACCTGGCGCTGGAGGACGGCGGGTCGGCA
                                                          1200
CACGGGGTGTTCCTGCTAAACAGCAATGCCATGGATGTGGTCCTGCAGCCGAGCCCTGCC
                                                          1260
CTTAGCTGGAGGTCGACAGGTGGGATCCTGGATGTCTACATCTTCCTGGGCCCAGAGCCC
                                                          1320
AAGAGCGTGGTGCAGCAGTACCTGGACGTTGTGGGATACCCGTTCATGCCGCCATACTGG
                                                          1380
GGCCTGGGCTTCCACCTGTGCCGCTGGGGCTACTCCTCCACCGCTATCACCCGCCAGGTG
                                                          1440
GTGGAGAACATGACCAGGGCCCACTTCCCCCTGGACGTCCAATGGAACGACCTGGACTAC
                                                          1500
ATGGACTCCCGGAGGGACTTCACGTTCAACAAGGATGGCTTCCGGGACTTCCCGGCCATG
                                                          1560
GTGCAGGAGCTGCACCAGGGCGGCCGGCGCTACATGATGATCGTGGATCCTGCCATCAGC
                                                          1620
AGCTCGGGCCCTGCCGGGAGCTACAGGCCCTACGACGAGGGTCTGCGGAGGGGGGTTTTC
                                                          1680
ATCACCAACGAGACCGGCCAGCCGCTGATTGGGAAGGTATGGCCCGGGTCCACTGCCTTC
                                                          1740
CCCGACTTCACCAACCCCACAGCCCTGGCCTGGTGGGAGGACATGGTGGCTGAGTTCCAT
                                                          1800
GACCAGGTGCCCTTCGACGGCATGTGGATTGACATGAACGAGCCTTCCAACTTCATCAGA
                                                          1860
GGCTCTGAGGACGGCTGCCCCAACAATGAGCTGGAGAACCCACCCTACGTGCCTGGGGTG
                                                          1920
GTTGGGGGGACCCTCCAGGCGGCCACCATCTGTGCCTCCAGCCACCAGTTTCTCTCCACA
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                                                        1980
CACTACAACCTGCACAACCTCTACGGCCTGACCGAAGCCATCGCCTCCCACAGGGCGCTG
                                                        2040
GTGAAGGCTCGGGGGACACGCCCATTTGTGATCTCCCGCTCGACCTTTGCTGGCCACGGC
                                                        2100
CGATACGCCGGCCACTGGACGGGGGACGTGTGGAGCTCCTGGGAGCAGCTCGCCTCCTCC
                                                        2160
GTGCCAGAAATCCTGCAGTTTAACCTGCTGGGGGTGCCTCTGGTCGGGGCCGACGTCTGC
                                                        2220
GGCTTCCTGGGCAACACCTCAGAGGAGCTGTGTGTGCGCTGGACCCAGCTGGGGGCCTTC
                                                        2280
TACCCCTTCATGCGGAACCACAACAGCCTGCTCAGTCTGCCCCAGGAGCCGTACAGCTTC
                                                        2340
AGCGAGCCGGCCCAGCAGGCCATGAGGAAGGCCCTCACCCTGCGCTACGCACTCCTCCCC
                                                        2400
CACCTCTACACACTGTTCCACCAGGCCCACGTCGCGGGGGAGACCGTGGCCCGGCCCCTC
                                                        2460
TTCCTGGAGTTCCCCAAGGACTCTAGCACCTGGACTGTGGACCACCAGCTCCTGTGGGGG
                                                        2520
GAGGCCCTGCTCATCACCCCAGTGCTCCAGGCCGGGAAGGCCGAAGTGACTGGCTACTTC
                                                        2580
CCCTTGGGCACATGGTACGACCTGCAGACGGTGCCAATAGAGGCCCTTGGCAGCCTCCCA
                                                        2640
CCCCCACCTGCAGCTCCCCGTGAGCCAGCCATCCACAGCGAGGGGCAGTGGGTGACGCTG
                                                        2700
CCGGCCCCCCTGGACACCATCAACGTCCACCTCCGGGCTGGGTACATCATCCCCCTGCAG
                                                        2760
GGCCCTGGCCTCACAACCACAGAGTCCCGCCAGCAGCCCATGGCCCTGGCTGTGGCCCTG
                                                        2820
ACCAAGGGTGGAGAGGCCCGAGGGGAGCTGTTCTGGGACGATGGAGAGAGCCTGGAAGTG
                                                        2880
CTGGAGCGAGGGGCCTACACACAGGTCATCTTCCTGGCCAGGAATAACACGATCGTGAAT
                                                        2940
GAGCTGGTACGTGTGACCAGTGAGGGAGCTGGCCTGCAGCTGCAGAAGGTGACTGTCCTG
                                                        3000
GGCGTGGCCACGGCGCCCCAGCAGGTCCTCTCCAACGGTGTCCCTGTCTCCAACTTCACC
                                                        3060
TACAGCCCCGACACCAAGGTCCTGGACATCTGTGTCTCGCTGTTGATGGGAGAGCAGTTT
                                                        3120
CTCGTCAGCTGGTGTTAGCCGGGCGGAGTGTGTTAGTCTCTCCAGAGGGAGGCTGGTTCC
                                                        3180
CCAGGGAAGCAGAGCCTGTGTGCGGGCAGCAGCTGTGTGCGGGCCTGGGGGTTGCATGTG
                                                        3240
TCACCTGGAGCTGGGCACTAACCATTCCAAGCCGCCGCATCGCTTGTTTCCACCTCCTGG
                                                        3300
GCCGGGGCTCTGGCCCCCAACGTGTCTAGGAGAGCTTTCTCCCTAGATCGCACTGTGGGC
                                                        3360
CGGGGCCTGGAGGGCTGCTCTGTGTTAATAAGATTGTAAGGTTTGCCCTCCTCACCTGTT
                                                        3420
GCCGGCATGCGGGTAGTATTAGCCACCCCCCTCCATCTGTTCCCAGCACCGGAGAAGGGG
                                                        3480
GTGCTCAGGTGGAGGTGTGGGGTATGCACCTGAGCTCCTGCTTCGCGCCTGCTGCTCTGC
                                                        3540
CCCAACGCGACCGCTTCCCGGCTGCCCAGAGGGCTGGATGCCTGCCGGTCCCCGAGCAAG
                                                        3600
CCTGGGAACTCAGGAAAATTCACAGGACTTGGGAGATTCTAAATCTTAAGTGCAATTATT
                                                3639
TTAATAAAAGGGGCATTTGGAATCAAAAAAAAAAAAAAA
```

Figure 3. Nucleotide sequence of the combined cDNA clones coding for acid α-glucosidase. The first in-frame start (ATG) and stop (TAG) codon are underlined, as well as the polyadenylation signal.

Figure 4. Amino acid sequence of acid α-glucosidase deduced from the cDNA sequence. The sequence starts at the first in-frame ATG and ends at the stop codon. The amino-terminal sequences obtained from the 110, 76 and 70 kD molecular forms of acid α-glucosidase are indicated. Tryptic and CNBr peptides are underlined. Possible glycosylation sites are indicated by an asterix.

polypeptide of 952 amino acid residues. The sequence around this ATG codon matches closely the consensus sequence for translation-initiation sites [(GCC)GCCA/GCCATGG] (Kozak, 1987). A polyadenylation signal (AATAAA) at position 3603 is followed 16 nt later by a stretch of adenosine residues. This indicates that the complete coding sequence and the entire 3' untranslated region (UTR) was cloned.

The amino acid sequence deduced from the open reading frame from position 220 to position 3076 is shown in Figure 4. Four of the 10 amino acids after the first methionine are basic. These are followed by a stretch of 27 amino acids, 20 of which are hydrophobic. A serine is located in this hydrophobic stretch. These features of the amino acid sequence fulfil the requirements for a signal peptide common to lysosomal and secretory proteins (Watson, 1984).

Sequences of several peptides either obtained by tryptic digestion or CNBr

cleavage of human placenta acid α-glucosidase were identified (underlined in Figure 4). The amino-terminal sequence of the 110 kD precursor isolated from human urine and the amino-terminal sequence of the 76 and 70 kD mature forms isolated from placenta were found to start at amino acids 70, 123 and 204, respectively. Seven possible glycosylation sites (Asn-X-Ser/Thr) are indicated by an asterix (Figure 4). The asparagine residues at positions 140 and 233 were not detected in the amino acid sequence analysis. All other asparagine residues in the various peptides gave a clear signal.

Amino acid homology

Comparison of the amino acid sequences of acid α-glucosidase, human isomaltase, and rabbit sucrase-isomaltase showed a remarkable homology between these proteins. Sucrase-isomaltase (EC 3.2.1.48-10) is an enzyme complex anchored in the apical membrane of intestinal epithelial cells. The complex is synthesized as a single chain precursor (prosucrase-isomaltase) which is proteolytically cleaved when exposed to pancreatic proteases in the intestinal lumen. The resulting sucrase and isomaltase subunits stay associated with one another in a complex by non-covalent linkages. They have different substrate specificities for sucrose and isomaltose respectively, but share activity for maltose (Brunner et al., 1979; Hauri et al., 1979; Montgomery et al., 1981; Spiess et al., 1982; for review see Semenza 1986). The two subunits have 41% of their amino acids identical when they are aligned (Hunziker et al., 1986). In Figure 5 we have aligned the amino acid sequence of acid α-glucosidase with the rabbit isomaltase and sucrase subunit (Hunziker et al., 1986), and the recently published partial sequence of the human isomaltase (Green et al., 1987). Identical amino acids in the sequences are given in boxes. They comprise 26.5% (252 out of 952). Clusters of identical amino acids occur throughout the sequence. The homology around positions 192, 316, 336, 445, 519 and 667 is most evident (Figure 5). Especially noteworthy are the identical sequences around aspartic acid at position 518, assigned to the active site of sucrase and isomaltase (Quaroni and Semenza, 1976; Hunziker et al., 1986). As much as 10 out of 13 amino acid residues in all four sequences are identical in the region surrounding this aspartic acid.

Comparison of the amino acid sequence of acid α-glucosidase with each of the other three proteins separately showed that acid α-glucosidase has 41% identical amino acid residues with human isomaltase, 40% with rabbit isomaltase and 36% with rabbit sucrase. When the nucleotides are aligned in the same way, the homology of acid α-

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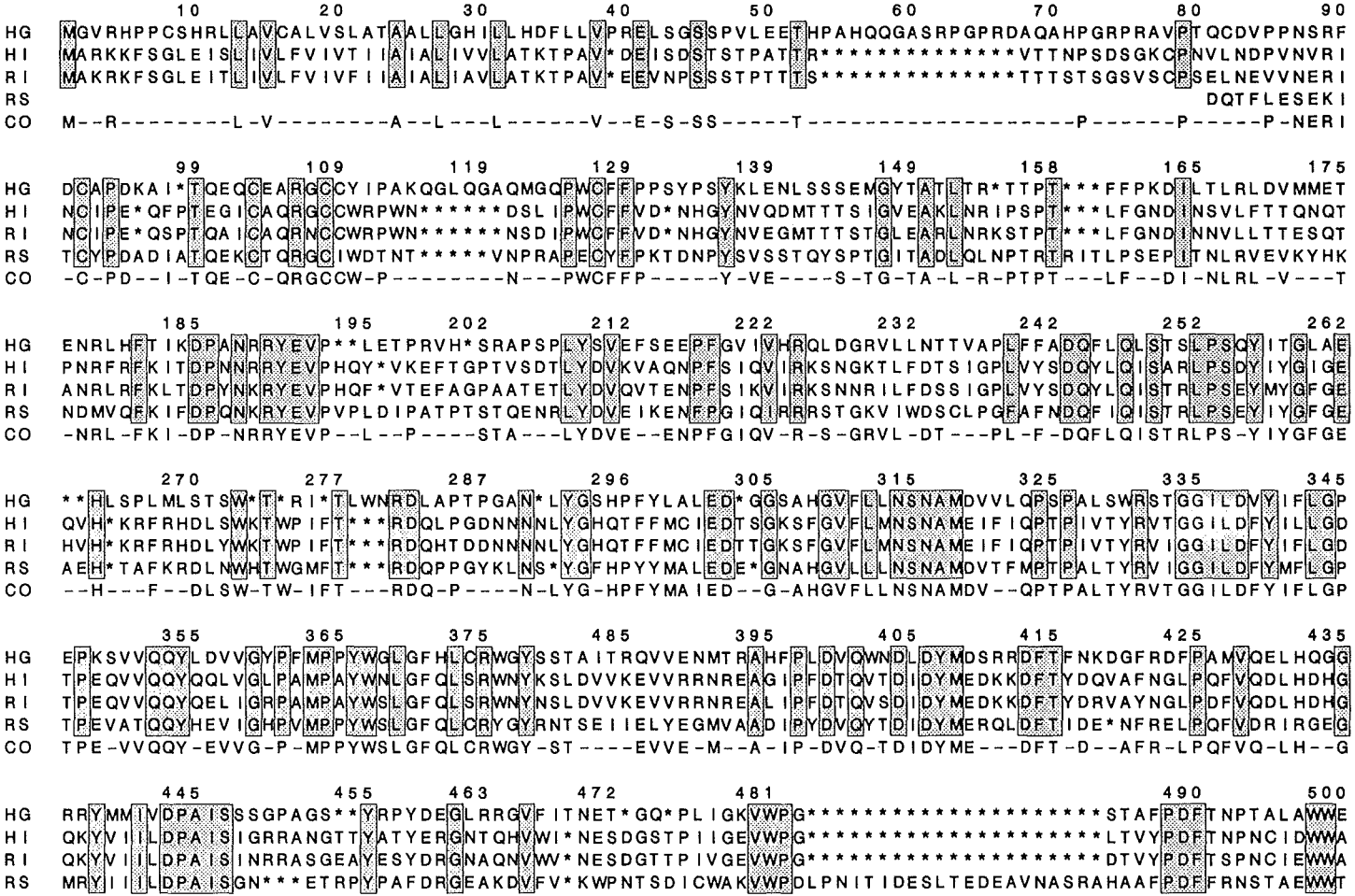
Figure 5. Amino acid homology of human acid α-glucosidase (HG), human isomaltase (HI), rabbit isomaltase (RI), and rabbit sucrase (RS). Boxes are placed around amino acids identical in all four sequences. Consensus sequence (CO): Amino acids identical in two or more sequences are indicated, whereby identical amino acids in human and rabbit isomaltase are counted as one.


10 20 30 40 50 60 70 80 90
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HI MARKKFSGLEISLIVLFVIVTIIAIALIVVLATKTPAV*DEISDSTSTPATTR**********VTTNPSDSGKCPNVLNDPVNVRI
RI MAKRKFSGLEITLIVLFVIVFIIAIALIAVLATKTPAV*EEVNPSSSTPTTTS**********TTTSTSGSVSCPSELNEVVNERI
RS DQTFLESEKI
CO M--R--------L-V--------A--L---L------V--E-S-SS-----T-------------------P------P-----P-NERI
99 109 119 129 139 149 158 165 175
HG DCAPDKAI*TQEQCEARGCCYIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTR*TTPT***FFPKDILTLRLDVMMET
HI NCIPE*QFPTEGICAQRGCCWRPWN******DSLIPWCFFVD*NHGYNVQDMTTTSIGVEAKLNRIPSPT***LFGNDINSVLFTTQNQT
RI NCIPE*QSPTQAICAQRNCCWRPWN******NSDIPWCFFVD*NHGYNVEGMTTTSTGLEARLNRKSTPT***LFGNDINNVLLTTESQT
RS TCYPDADIATQEKCTQRGCIWDTNT*****VNPRAPECYFPKTDNPYSVSSTQYSPTGITADLQLNPTRTRITLPSEPITNLRVEVKYHK
CO -C-PD--I-TQE-C-QRGCCW-P--------N---PWCFFP-----Y-VE----S-TG-TA-L-R-PTPT---LF--DI-NLRL-V---T
185 195 202 212 222 232 242 252 262
HG ENRLHFTIKDPANRRYEVP**LETPRVH*SRAPSPLYSVEFSEEPFGVIVHRQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAE
HI PNRFRFKITDPNNRRYEVPHQY*VKEFTGPTVSDTLYDVKVAQNPFSIQVIRKSNGKTLFDTSIGPLVYSDQYLQISARLPSDYIYGIGE
RI ANRLRFKLTDPYNKRYEVPHQF*VTEFAGPAATETLYDVQVTENPFSIKVIRKSNNRILFDSSIGPLVYSDQYLQISTRLPSEYMYGFGE
RS NDMVQFKIFDPQNKRYEVPVPLDIPATPTSTQENRLYDVEIKENFPGIQIRRRSTGKVIWDSCLPGFAFNDQFIQISTRLPSEYIYGFGE
CO -NRL-FKI-DP-NRRYEVP--L--P----STA---LYDVE--ENPFGIQV-R-S-GRVL-DT---PL-F-DQFLQISTRLPS-YIYGFGE
270 277 287 296 305 315 325 335 345
HG **HLSPLMLSTSW*T*RI*TLWNRDLAPTPGAN*LYGSHPFYLALED*GGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGP
HI QVH*KRFRHDLSWKTWPIFT***RDQLPGDNNNNLYGHQTFFMCIEDTSGKSFGVFLMNSNAMEIFIQPTPIVTYRVTGGILDFYILLGD
RI HVH*KRFRHDLYWKTWPIFT***RDQHTDDNNNNLYGHQTFFMCIEDTTGKSFGVFLMNSNAMEIFIQPTPIVTYRVIGGILDFYIFLGD
RS AEH*TAFKRDLNWHTWGMFT***RDQPPGYKLNS*YGFHPYYMALEDE*GNAHGVLLLNSNAMDVTFMPTPALTYRVIGGILDFYMFLGP
CO --H---F--DLSW-TW-IFT---RDQ-P----N-LYG-HPFYMAIED--G-AHGVFLLNSNAMDV--QPTPALTYRVTGGILDFYIFLGP
355 365 375 485 395 405 415 425 435
HG EPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGG
HI TPEQVVQQYQQLVGLPAMPAYWNLGFQLSRWNYKSLDVVKEVVRRNREAGIPFDTQVTDIDYMEDKKDFTYDQVAFNGLPQFVQDLHDHG
RI TPEQVVQQYQELIGRPAMPAYWSLGFQLSRWNYNSLDVVKEVVRRNREALIPFDTQVSDIDYMEDKKDFTYDRVAYNGLPDFVQDLHDHG
RS TPEVATQQYHEVIGHPVMPPYWSLGFQLCRYGYRNTSEIIELYEGMVAADIPYDVQYTDIDYMERQLDFTIDE*NFRELPQFVDRIRGEG
CO TPE-VVQQY-EVVG-P-MPPYWSLGFQLCRWGY-ST----EVVE-M--A-IP-DVQ-TDIDYME---DFT-D--AFR-LPQFVQ-LH--G
445 455 463 472 481 490 500
HG RRYMMIVDPAISSSGPAGS**YRPYDEGLRRGVFITNET*GQ*PLIGKVWPG**********STAFPDFTNPTALAWWE
HI QKYVIILDPAISIGRRANGTTYATYERGNTQHVWI*NESDGSTPIIGEVWPG**********LTVYPDFTNPNCIDWWA
RI QKYVIILDPAISINRRASGEAYESYDRGNAQNVWV*NESDGTTPIVGEVWPG**********DTVYPDFTSPNCIEWWA
RS MRYIIILDPAISGN***ETRPYPAFDRGEAKDVFV*KWPNTSDICWAKVWPDLPNITIDESLTEDEAVNASRAHAAFPDFFRNSTAEWWT
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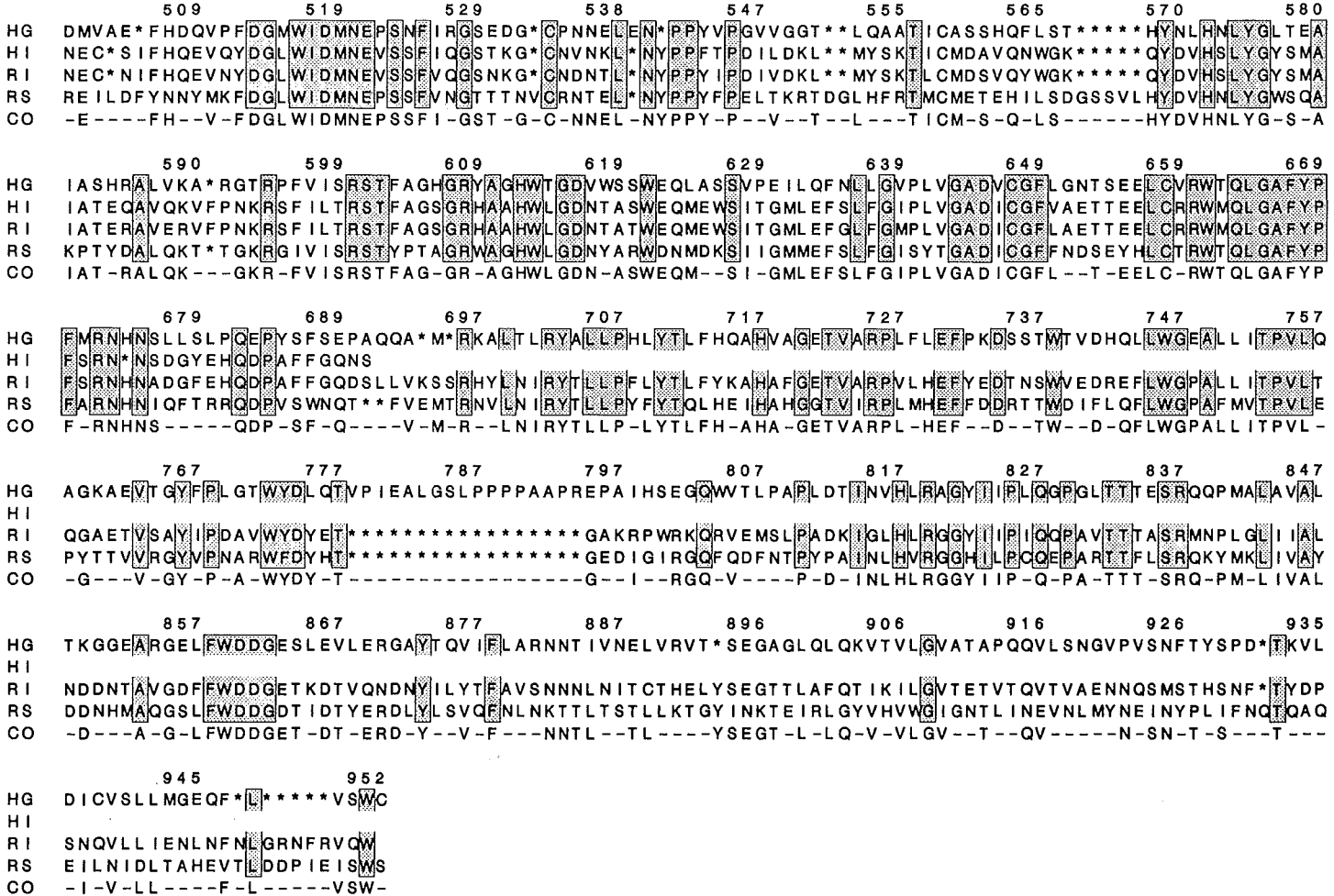
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HG DMVAE*FHDQVPFDGMWIDMNEPSNFIRGSEDG*CPNNELEN*PPYVPGVVGGT**LQAATICASSHQFLST*****HYNLHNLYGLTEA
HI NEC*SIFHQEVQYDGLWIDMNEVSSFIQGSTKG*CNVNKL*NYPPFTPDILDKL**MYSKTICMDAVQNWGK*****QYDVHSLYGYSMA
RI NEC*NIFHQEVNYDGLWIDMNEVSSFVQGSNKG*CNDNTL*NYPPYIPDIVDKL**MYSKTLCMDSVQYWGK*****QYDVHSLYGYSMA
RS REILDFYNNYMKFDGLWIDMNEPSSFVNGTTTNVCRNTEL*NYPPYFPELTKRTDGLHFRTMCMETEHILSDGSSVLHYDVHNLYGWSQA
CO -E----FH--V-FDGLWIDMNEPSSFI-GST-G-C-NNEL-NYPPY-P--V--T--L---TICM-S-Q-LS------HYDVHNLYG-S-A
590 599 609 619 629 639 649 659 669
HG IASHRALVKA*RGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYP
HI IATEQAVQKVFPNKRSFILTRSTFAGSGRHAAHWLGDNTASWEQMEWSITGMLEFSLFGIPLVGADICGFVAETTEELCRRWMQLGAFYP
RI IATERAVERVFPNKRSFILTRSTFAGSGRHAAHWLGDNTATWEQMEWSITGMLEFGLFGMPLVGADICGFLAETTEELCRRWMQLGAFYP
RS KPTYDALQKT*TGKRGIVISRSTYPTAGRWAGHWLGDNYARWDNMDKSIIGMMEFSLFGISYTGADICGFFNDSEYHLCTRWTQLGAFYP
CO IAT-RALQK---GKR-FVISRSTFAG-GR-AGHWLGDN-ASWEQM--SI-GMLEFSLFGIPLVGADICGFL--T-EELC-RWTQLGAFYP
679 689 697 707 717 727 737 747 757
HG FMRNHNSLLSLPQEPYSFSEPAQQA*M*RKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLQ
HI FSRN*NSDGYEHQDPAFFGQNS
RI FSRNHNADGFEHQDPAFFGQDSLLVKSSRHYLNIRYTLLPFLYTLFYKAHAFGETVARPVLHEFYEDTNSWVEDREFLWGPALLITPVLT
RS FARNHNIQFTRRQDPVSWNQT**FVEMTRNVLNIRYTLLPYFYTQLHEIHAHGGTVIRPLMHEFFDDRTTWDIFLQFLWGPAFMVTPVLE
CO F-RNHNS-----QDP-SF-Q----V-M-R--LNIRYTLLP-LYTLFH-AHA-GETVARPL-HEF--D--TW--D-QFLWGPALLITPVL-
767 777 787 797 807 817 827 837 847
HG AGKAEVTGYFPLGTWYDLQTVPIEALGSLPPPPAAPREPAIHSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVAL
HI
RI QGAETVSAYIPDAVWYDYET**********GAKRPWRKQRVEMSLPADKIGLHLRGGYIIPIQGPAVTTTASRMNPLGLIIAL
RS PYTTVVRGYVPNARWFDYHT**********GEDIGIRGQFQDFNTPYPAINLHVRGGHILPCQEPARTTFLSRQKYMKLIVAY
CO -G---V-GY-P-A-WYDY-T----------G--I--RGQ-V----P-D-INLHLRGGYIIP-Q-PA-TTT-SRQ-PM-LIVAL
857 867 877 887 896 906 916 926 935
HG TKGGEARGELFWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVT*SEGAGLQLQKVTVLGVATAPQQVLSNGVPVSNFTYSPD*TKVL
HI
RI NDDNTAVGDFFWDDGETKDTVQNDNYILYTFAVSNNNLNITCTHELYSEGTTLAFQTIKILGVTETVTQVTVAENNQSMSTHSNF*TYDP
RS DDNHMAQGSLFWDDGDTIDTYERDLYLSVQFNLNKTTLTSTLLKTGYINKTEIRLGYVHVWGIGNTLINEVNLMYNEINYPLIFNQTQAQ
CO -D---A-G-LFWDDGET-DT-ERD-Y--V-F---NNTL--TL----YSEGT-L-LQ-V-VLGV--T--QV-----N-SN-T-S---T---
945 952
HG DICVSLLMGEQF*L*****VSWC
HI
RI SNQVLLIENLNFNLGRNFRVQW
RS EILNIDLTAHEVTLDDPIEISWS
CO -I-V-LL----F-L-----VSW-

glucosidase with human isomaltase, rabbit isomaltase and rabbit sucrase is 44%, 44%, and 40% respectively.

The plot in Figure 6, made with Diagon Software (Staden, 1982), illustrates the high homology between α-glucosidase and the other proteins when not only identical amino acids but also conservative changes are taken into account.

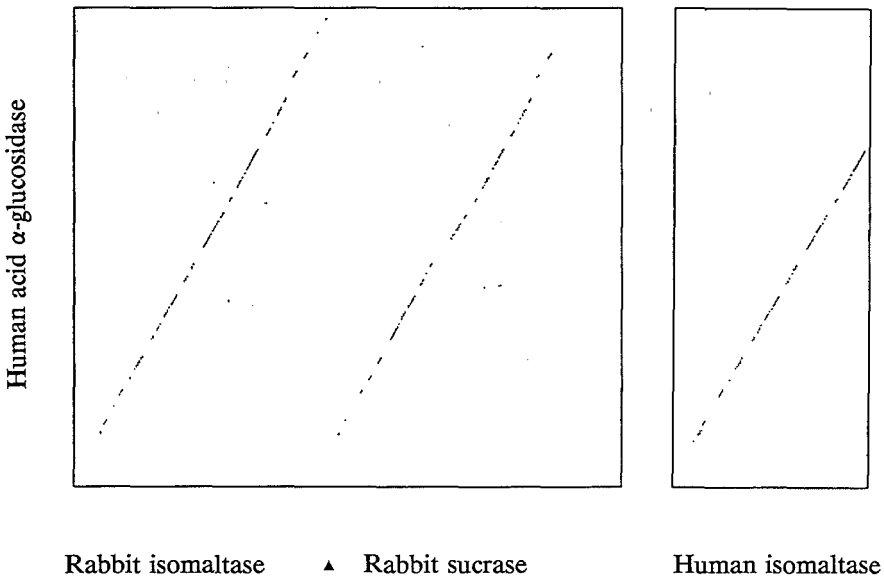

Figure 6. Comparison of amino acid sequences of acid α-glucosidase, rabbit sucrase-isomaltase, and human isomaltase when Dayhoff "interchanges" are taken into account. Plots were made using Diagon software. (The span was 11 amino acids, the threshold was set at 145. The matrix used was MDM78 from Dayhoff (Staden, 1982)).

Discussion

We have isolated cDNA clones encoding human acid α-glucosidase. The combined length of the clones is 3.6 kb, containing the entire coding sequence. The identity of the cDNA was established in several ways. The clone hybridized with DNA sequences from human chromosome 17, particularly with those derived from a small and cytogenetically undetectable chromosomal fragment carrying the acid α-glucosidase gene. Furthermore, the clone hybridized with a messenger of 3.6 kb, which was absent in cells of two patients with infantile glycogenosis type II. The

unambiguous proof was the identification of the amino-terminal sequences of the 110 kD precursor, the 76 and 70 kD mature forms of acid α-glucosidase, and several tryptic and CNBr peptides in the amino acid sequence deduced from the cDNA clone. These sequences are all encoded in the longest open reading frame, in which only one methionine precedes the amino terminus of the 110 kD precursor. That translation starts, indeed, at this methionine is further indicated by the fact that the ATG codon is flanked by the consensus sequence for translation-initiation sites, and is followed by a putative signal sequence. At the 3' end of the clone a polyadenylation signal is followed by a stretch of adenosine residues. This implies that the complete coding sequence and the 3' UTR of acid α-glucosidase was cloned. Since the combined length of the cDNA is similar to the estimated length of the mRNA, we might have cloned the entire 5' UTR as well, although it is possible that few nucleotides are missing at the utmost 5' end.

The cDNA clone recognized restriction fragments in human placenta DNA similar in size to the fragments detected by Martiniuk et al. (1986) with their partial acid α-glucosidase cDNA. A 20 kb human *Eco*RI fragment was also observed to hybridize with an acid α-glucosidase cDNA cloned from monkey testis (Konings et al., 1984). However, we have found no cross-hybridization of this previously cloned monkey cDNA and the presently described human cDNA, casting doubt on the identity of the monkey cDNA.

Analysis of DNA and RNA extracted from fibroblasts of patients showed deficiency of mRNA in some cases, but did not reveal gene deletions.

The human cDNA encodes a protein with a molecular weight of 105.3 kD, similar in size to the 100 kD unglycosylated precursor of acid α-glucosidase which is produced by *in vitro* translation (Van der Horst et al., 1987).

Some interesting features concerning the post-translational processing of acid α-glucosidase emerge when the spacing of the sequences coding for the precursor and mature enzyme are considered. The 110 kD amino acid sequence starts 69 amino acids after the first methionine. Since signal peptides are in general not much longer than 30 amino acids (Watson, 1984), the 110 kD precursor isolated from human urine may not be the first *in vivo* precursor. It is likely that some proteolytic processing has occurred after removal of the signal peptide. Indeed, as described by Oude Elferink et al. (1984), the precursor present in human urine is slightly different from the precursor in fibroblasts. The precursor found in fibroblasts might well be the first glycosylated precursor, missing only the signal peptide.

The distance between the amino termini of the 110 kD precursor and the 76 kD mature form of acid α-glucosidase is only 53 amino acids (5.5 kD). This implies that the bulk of proteolytic processing necessary to reduce 110 kD to 76 kD occurs at the carboxy-terminal end. The 110 kD precursor starting at position 70 in the amino acid sequence has a calculated molecular weight of 97 kD, when not glycosylated. The 76

kD mature form is glycosylated and contains probably four sugar chains (Belen'ky et al., 1979; Mutsaers et al., 1987), which account for ~5 kD. This implies that a fragment of ~20 kD (97-71-5.5) is spliced off at the carboxy-terminal end. Other lysosomal enzymes known to undergo carboxy-terminal processing are cathepsin D and ß-glucuronidase (Erickson et al., 1984). However, their carboxy-terminal processing is limited to only 1 kD for cathepsin D and ~3 kD for ß-glucuronidase.

The difference between the starting points of the 76- and 70 kD mature species is 78 amino acid residues, or 8.0 kD. This suggests that processing of the 76 kD mature enzyme occurs mainly at the amino-terminal end. Carboxy-terminal sequencing is required to determine the exact end of the mature forms of acid α-glucosidase.

The predicted amino acid sequence between the 76 kD amino-terminus and the carboxy-terminal end of the protein contains seven possible sites of N-linked glycosylation. But only four carbohydrate chains are present in the 76- and 70 kD enzymes. It is likely that the asparagine residues at position 140 and 233 are glycosylated since they were not detected by amino acid sequencing carried out twice. By contrast, all other asparagine residues present in the peptides were detected by amino acid sequencing. The site at position 140 is lost by amino-terminal processing of the 76 kD to the 70 kD form. The two potential glycosylation sites at position 882 and 925 are lost during carboxy-terminal processing from the 110 kD to the 76 kD form. This implies that two of the three remaining glycosylation sites at positions 390, 470, and 652 are used.

The amino acid sequence of human acid α-glucosidase showed a remarkable homology with the amino acid sequences of rabbit sucrase-isomaltase and human isomaltase. A comparison of the acid α-glucosidase sequence with the protein sequence data library of the National Biochemical Research Foundation (NBRF) revealed no significant homologies with other proteins. The degree of homology between acid α-glucosidase, sucrase and isomaltase is surprisingly high considering the different characteristics of these proteins, although some homology could have been anticipated because of their similar substrate specificities. Acid α-glucosidase is a soluble lysosomal enzyme with optimal activity at a pH between 4 and 5 (Jeffrey et al., 1970a, 1970b; Palmer, 1971a, 1971b; Rosenfeld, 1975; Koster and Slee, 1977) whereas sucrase-isomaltase is anchored in the apical membrane of intestinal epithelial cells and has optimal activity at neutral pH (for review, see Semenza, 1986). Moreover, glycogen is the natural substrate of the lysosomal enzyme, while sucrase-isomaltase does not act on this polysaccharide.

As proposed by Hunziker et al. (1986), the isomaltase-sucrase single chain precursor (prosucrase-isomaltase) has arisen by duplication of an ancestral isomaltase gene. Subsequently, the two parts of the new protein evolved into one part still acting on isomaltose, whereas the other acquired activity towards sucrose. The duplication supposedly took place before separation of mammals and reptiles, e.i. more than 3 x

10^8 years ago. The fact that the partial sequence of human isomaltase has a much higher homology with the rabbit counterpart (85% at the DNA level and 82% at the amino acids level) (Green et al., 1987) supports this hypothesis, since the separation between man and rodents took place only 8 x 10^7 years ago. Since lysosomal α-glucosidase has an equal degree of homology with isomaltase and sucrase, and acid α-glucosidase is not the product of a duplicated gene, we suggest acid α-glucosidase evolved from the same ancestral gene before or at the time of the gene duplication.

A partial amino acid sequence of the ancestral protein was postulated by choosing those amino acids at each position that occur most frequently in the four aligned proteins (Figure 5). Identical amino acids occurring in the human and rabbit isomaltase sequences were counted as one. This consensus sequence comprises 63% (595 out of 951) of the shared amino acid positions.

The homology between acid α-glucosidase, sucrase and isomaltase allows us to draw some conclusions about the functionally important domains. For instance, the stretch of six identical amino acid residues at positions 557 to 562 includes the aspartic acid residue that is a constituent of the active site of sucrase and isomaltase (Quaroni and Semenza, 1976; Hunziker et al., 1986). This strongly suggests that these sequences are also part of the active site of acid α-glucosidase. Stretches of identical amino acids occurring at various other positions may be of additional importance for recognition and/or binding of substrate.

Acid α-glucosidase and prosucrase-isomaltase differ most at the amino-terminal and carboxy-terminal ends. Different amino acids are present in the signal peptides of both proteins, and, indeed, they channel the enzymes to a different cellular location. Furthermore, the signal peptide of prosucrase-isomaltase is not cleaved off and serves as a membrane anchor (Hunziker et al., 1986), whereas acid α-glucosidase, like other lysosomal enzymes, loses its signal peptide. The serine and threonine-rich segment of prosucrase-isomaltase, which forms a connecting stalk between the transmembrane and other domains of sucrase-isomaltase, is absent in acid α-glucosidase. Other non-homologous regions at the amino-terminal and carboxy-terminal ends may have a function for enzyme-specific post-translational processing or may determine the partially different substrate specificities. Computer-assisted comparison of the sequences may reveal further interesting similarities and dissimilarities. Analyses of mutations in acid α-glucosidase in various cases of glycogenosis type II will give additional information on which sequences and domains are important for post-translational processing and catalytic function.

Materials and methods

Isolation of cDNA clones

The following cDNA libraries were used. A human testis λgt11 cDNA library was purchased from Clontech Inc., (Palo Alto, CA) with 1 x 10^6 independent clones and insert lengths between 0.7 and 3.3 kb.

A human placenta λgt11 cDNA library (VII-75-1, placenta Al) was a generous gift of Dr J.E. Sadler (Washington University School of Medicine, St. Louis, MO). The library consisted of 4 x 10^6 independent clones with insert lengths varying between 1.0 and 3 kb (Ye et al., 1987).

Screening of libraries

Libraries were screened with antibodies essentially as described (Huynh et al., 1985). A polyclonal antiserum raised in rabbit against purified human placenta acid α-glucosidase was used. The IgG fraction was isolated, and antibodies against E.coli and phage antigens were absorbed by incubating the antiserum with nitrocellulose filters soaked in lysates of Y1089 containing lysogenic λgt11. Screening of libraries with nucleic acid probes was as described (Benton and Davis, 1977). A partial restriction map of the isolated clones is given in Figure 7.

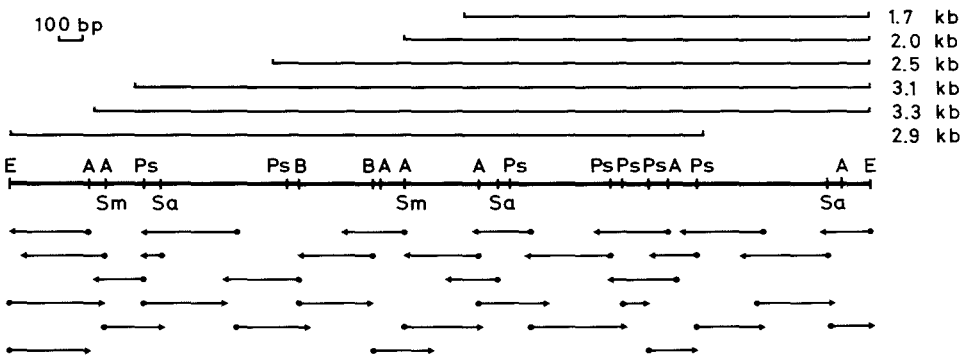

Figure 7. Cloning and sequencing strategy for acid α-glucosidase. Six isolated clones are indicated. Bottom line: partial restriction map of the combined clones. The 1.7 and 2.0 kb clones were isolated from a human testis library using antibody-screening, and the 2.0 kb clone was used to isolate the 2.5, 2.9, 3.1 and 3.3 kb clones from a human placenta library. Arrows indicate the sequence strategy followed. Ps=PstI, A=AvaI, E=EcoRI, Sa=SacI, Sm=SmaI.

Isolation and analysis of cellular DNA and RNA

Standard procedures were used for extraction of DNA and Southern blot analysis (Maniatis et al., 1982). Cellular RNA was isolated using the lithium chloride method (Auffray and Rougeon 1980). Total RNA was fractionated on a 1% agarose gel in the presence of formaldehyde and blotted onto nitrocellulose filters. Filters were hybridized in the standard hybridization buffer (Maniatis et al., 1982). After 16 hours the filters were washed till a stringency of 0.3xSSC, and an autoradiograph was made. DNA probes were radioactively labelled as described by Feinberg and Vogelstein (1983).

Sequence analyses of DNA, protein and peptides

Sequencing of cDNA clones was done by subcloning appropriate fragments in pTZ18 or pTZ19 (Pharmacia), using the dideoxy chain termination method with ^{35}SdATP as labelling component (Biggin et al., 1983). The strategy used for cDNA sequencing is outlined in Figure 7. When no appropriate

fragments could be obtained, an oligonucleotide complementary to the cDNA was used as a primer. Oligonucleotides were 22 nucleotides long, and synthesized on an Applied Biosystems 381A DNA synthesizer. Purification was according the manufacturers protocol.

Precursor acid α-glucosidase (110 kD) was purified from human urine as described (Oude Elferink et al., 1984) and analyzed for purity by polyacrylamide gel electrophoresis (PAGE) in the presence of sodiumdodecylsulfate (SDS) according to Laemmli (Laemmli 1970). The 76 kD and 70 kD mature forms of acid α-glucosidase were purified from human placenta as described previously (Reuser et al. 1985). These two species (3 mg) were electrophoretically separated using SDS-PAGE (10% gel). The SDS was purchased from Serva (no.20760) and recrystallized as described (Hunkapillar et al. 1983). Thioglycolate (0.1 mM) was added to the cathodal buffer compartment. The gel was incubated in 4 M sodium acetate to visualize the protein. Protein bands were excised and sliced into pieces from which the protein was eluted electrophoretically using a Schleicher and Schuell "BIOTRAP". The buffer used was 10 mM ammoniumbicarbonate (pH 8.2) with addition of 0.01% SDS and 0.05% ß-mercaptoethanol. Electroelution was performed for 5 h at room temperature followed by 1 h in the same buffer without SDS. Electroeluted protein was analyzed by SDS-PAGE and used for determination of amino-terminal sequences. Amino-terminal sequences of the 76 kD and 70 kD species were also obtained after separation on SDS- PAGE followed by electrophoretic transfer to polybrene coated glass-fiber sheets as described (Vandekerckhove et al., 1985).

Tryptic peptides were obtained from 2 mg of acid α-glucosidase purified from human placenta, containing equal amounts of 76 kD and 70 kD protein. After reduction and alkylation, the protein was digested with trypsin (Cooper Biomedical, no.3740) in 0.5 M Tris-HCl (pH 8.5), at 37 °C for 48 h using an enzyme-protein ratio of 3 to 100 (w/w) (Yuan et al., 1982). The resulting peptides were lyophilized and dissolved in acetonitrile (16%), and water (84%) containing 0.1% trifluoroacetic acid, and separated on a 25 cm C8 reversed-phase HPLC column (RP8 Lichrospher, Merck) using a gradient of 0-33% acetonitrile in water with 0.1% trifluoroacetic acid. Peaks were collected and rerun on a 25 cm μBondapak column (Waters Associates) using the same solvent system. Seemingly homogeneous fractions were collected and used for amino acid sequence analysis.

CNBr cleavage of placental acid α-glucosidase was carried out as described (O'Dowd et al., 1985). The resulting fragments were separated by SDS-PAGE (13% gel), and electrophoretically transferred to polybrene coated glass-fiber sheets for sequencing purpose. Amino acid sequence analysis of proteins and peptides was performed on an Applied Biosystems sequencer (477A) on line with the PTH (120A) analyzer.

Hybrid cell lines.

Human-hamster somatic cell hybrids were obtained by fusing thymidine kinase deficient (TK^-) Chinese hamster fibroblasts (cell line A23) with human leukocytes. Hybrid clones (17CB18A and 17CB26B) containing human chromosome 17 were selected in HAT medium (Littlefield, 1964) and investigated cytogenetically. The two independent clones containing no other human chromosomes than chromosome 17 were kindly provided by Dr. A. Geurts van Kessel, Dept. of Cell Biology and Genetics, Erasmus University Rotterdam.

A clone (60C2HAT) of the mouse LTK^- cell line containing a cytogenetically undetectable fragment of human chromosome 17 including the loci for thymidine kinase (TK), galactokinase (GALK) and acid α-glucosidase (GAA) was obtained by (HeLa) chromosome mediated gene transfer followed by HAT selection. The clone expressed human acid α-glucosidase activity. Through growth of 60C2HAT in medium containing BUdR a new clone was obtained that did no longer contain human chromosome sequences as jugded by the loss of activity for acid α-glucosidase and TK (60C2BUdR)(De Jonge et al., 1985).

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CHAPTER 5

Expression and routeing of human lysosomal α-glucosidase in transiently transfected mammalian cells[1]

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Summary

Previously isolated lysosomal α-glucosidase cDNA clones were ligated to full-length constructs for expression *in vitro* and in mammalian cells. One of these constructs (pSHAG1) did not code for functional enzyme, due to an arginine residue instead of a tryptophan residue at amino acid position 402. The mutation does not affect the rate of enzyme synthesis, but interferes with post-translational modification and intracellular transport of the acid α-glucosidase precursor. Using immuno-cytochemistry it is demonstrated that the mutant precursor traverses the endoplasmic reticulum and the Golgi complex, but does not reach the lysosomes. Pulse-chase experiments suggest premature degradation. The Trp^{402} containing enzyme (encoded by construct pSHAG2) is processed properly, and has catalytic activity. A fraction of the enzyme is localized at the plasma membrane. It is hypothesized that membrane association of the acid α-glucosidase precursor, as demonstrated by Triton X-114 phase separation, is responsible for transport to this location. Transiently expressed acid α-glucosidase also enters the secretory pathway, since a catalytically active precursor is found in the culture medium. This precursor has the proper characteristics for use in enzyme replacement therapy. Efficient uptake via the mannose 6-phosphate receptor results in degradation of lysosomal glycogen in cultured fibroblasts and muscle cells from patients with glycogenosis type II.

Introduction

Human acid α-glucosidase (glucan 1,4-α-glucosidase; EC 3.2.1.3) is a typical lysosomal enzyme with substrate specificity for glycogen, maltose and isomaltose (Rosenfeld, 1975). The enzyme is produced as a glycosylated 110 kD precursor, and is

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[1]Adapted from Biochem. J. **272**:485-492 (1990)

routed from the endoplasmic reticulum through the Golgi complex to the lysosomes (Hasilik & Neufeld, 1980a; Reuser et al., 1985). Phosphorylation of the precursor has been demonstrated (Hasilik and Neufeld, 1980b; Reuser et al., 1985) and transport of the enzyme to the lysosomes is considered to be mediated by the mannose 6-phosphate receptor. The proteolytic processing of the acid α-glucosidase precursor involves removal of both N- and C-terminal peptides of significant length (Hoefsloot et al., 1988) and leads to the formation of mature enzyme of 76 and 70 kD. A molecular species of 95 kD has been identified as a processing intermediate (Hasilik and Neufeld, 1980a; Oude Elferink et al., 1985; Reuser et al., 1985).

About a dozen different defects have been discovered in the synthesis, the intracellular transport and the function of mutant acid α-glucosidase in cells from patients with an inherited enzyme deficiency (Beratis et al., 1983; Reuser *et al.* 1987). The condition is known as glycogenosis type II, and leads to fatal heart and skeletal muscle dysfunction (Hers, 1963).

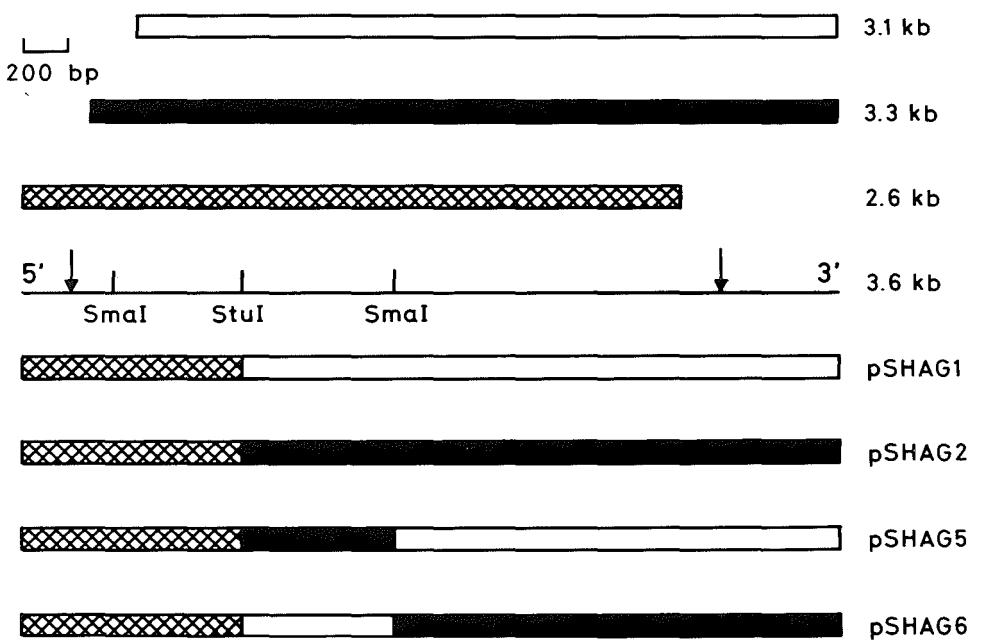

Figure 1. Acid α-glucosidase cDNA clones, and constructs. Depicted are cDNA clones (3.1 kb, 3.3 kb, 2.6 kb) (Hoefsloot et al., 1988) and constructs in pSG5 (pSHAG1, -2, -5,-6). cDNA constructs containing the entire coding sequence of acid α-glucosidase were obtained by fusing the 5' end of the 2.6 kb clone to the 3' ends of the 3.1 kb clone (pSHAG1) or the 3.3 kb clone (pSHAG2) at the unique StuI site. pSHAG5 and pSHAG6 were derived from pSHAG1 and pSHAG2 respectively by exchanging the internal SmaI-fragments. The arrows in the partial restriction map of the entire cDNA indicate the

Cloning of acid α-glucosidase cDNA has led to the unexpected finding that lysosomal α-glucosidase has probably arisen from the same ancestral gene as sucrase and isomaltase (Hoefsloot et al., 1988). The latter two enzymes are derived from a single 260 kD precursor by proteolytic cleavage. The enzyme complex is anchored in the apical membrane of intestinal epithelial cells via the uncleaved signal peptide of prosucrase-isomaltase (Hunziker et al., 1986; Semenza, 1989).

In this study we report on the expression of full length acid α-glucosidase cDNA constructs *in vitro* and in transfected mammalian cells. Immuno-electron microscopy and biochemical analyses were combined to investigate the intracellular routing and processing of acid α-glucosidase. Furthermore, the possible use of cDNA encoded acid α-glucosidase for enzyme replacement therapy was tested with fibroblasts and muscle cells from patients with glycogenosis type II.

Results

Translation in vitro

cDNA constructs containing the complete coding sequence for human acid α-

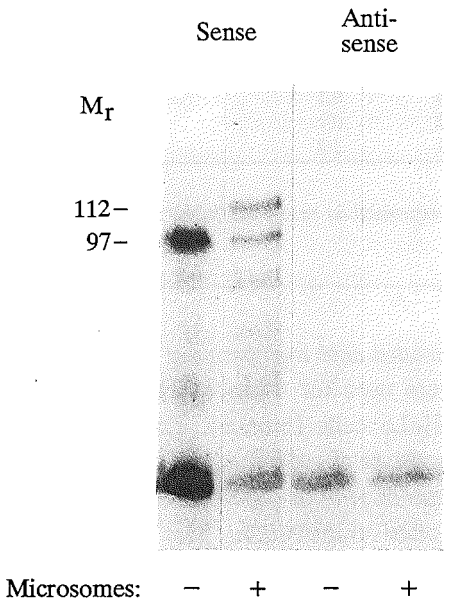

Figure 2. In vitro synthesis of human acid α-glucosidase. pSHAG2 (sense) and pSHAG2a (anti-sense) derived RNA was translated in a rabbit reticulocyte lysate in the absence (—) or presence (+) of canine pancreatic microsomes. Products were labelled with [^{35}S]methionine and separated by SDS-PAGE. The relative molecular masses of the unglycosylated and glycosylated acid α-glucosidase precursor are

glucosidase (Figure 1) were cloned in the eukaryotic expression vector pSG5 (Green et al, 1988). mRNA was synthesized from the T7 promoter in front of the cDNA insert, and used to direct protein synthesis in a reticulocyte translation system. Figure 2 shows the products obtained by transcription and translation of pSHAG2 (sense) and pSHAG2a (antisense). Only translation of sense mRNA led to protein production. The proteins formed in the absence and in the presence of microsomes corresponded in their molecular mass to the previously identified unglycosylated and glycosylated precursors of acid α-glucosidase obtained by *in vitro* translation of total RNA isolated from human fibroblasts (Van der Horst et al., 1987). Identical results were obtained with the other cDNA constructs depicted in Figure 1.

Table 1. Acid α-glucosidase activity in transfected cells

		Acid α-glucosidase activity					
		48 h		72 h		96 h	
Cell type	transfected construct	Cells	Medium	Cells	Medium	Cells	Medium
COS	nt	50.2	0.2	61.1	0.3	52.5	0.6
	pGA293	14.2	0.2	22.0	0.5	32.2	1.1
	pSHAG2	30.5	1.5	112.3	23.6	187.2	57.8
	pSHAG1					20.1	0.5
	pSHAG5					174.2	64.4
	pSHAG6					36.6	0.9
HeLa	nt	4.5	0.1				
	pGA293	2.2	0.1				
	pSHAG2	26.1	3.2				

nt, untransfected cells
Acid α-glucosidase activity is expressed as nmol MU/h per mg of protein

Functional analysis of cDNA encoded acid α-glucosidase

pSHAG1 and pSHAG2 were used for transient expression of acid α-glucosidase in COS cells (monkey) and HeLa cells (human). Transfection with a bacterial ß-galactosidase cDNA (pGA293, An et al., 1982) served as a negative control. Table 1 shows that the acid α-glucosidase activity of pSHAG2-transfected COS cells increased between 48 and 96 hours after transfection. The resulting enzyme level was approximately 5 times higher than in pGA293 transfected cells. The maximal activity in transfected HeLa cells was reached at 48 hours after transfection, and was much lower than the maximal activity in COS cells. Transfection of both cell types with pSHAG2 led to a dramatic increase of acid α-glucosidase activity in the culture media.

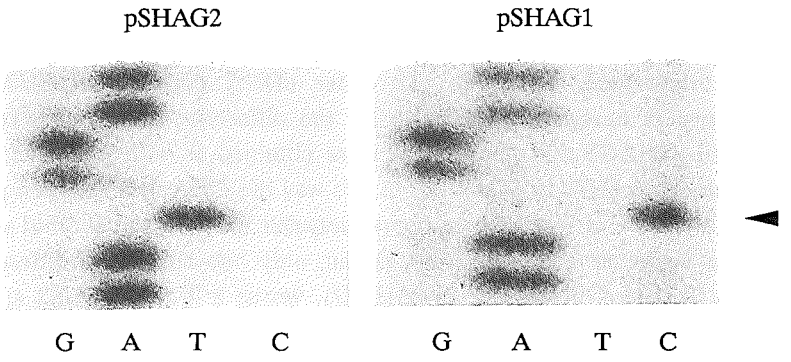

Figure 3. Nucleotide sequences of pSHAG1 and pSHAG2 cDNA inserts between nucleotides 1421 and 1427. Sequence reactions were performed using double stranded plasmid DNA as a template and a cDNA specific 21-mer (nucleotides 1329-1349) as the primer. The mutated nucleotide is indicated with an arrowhead.

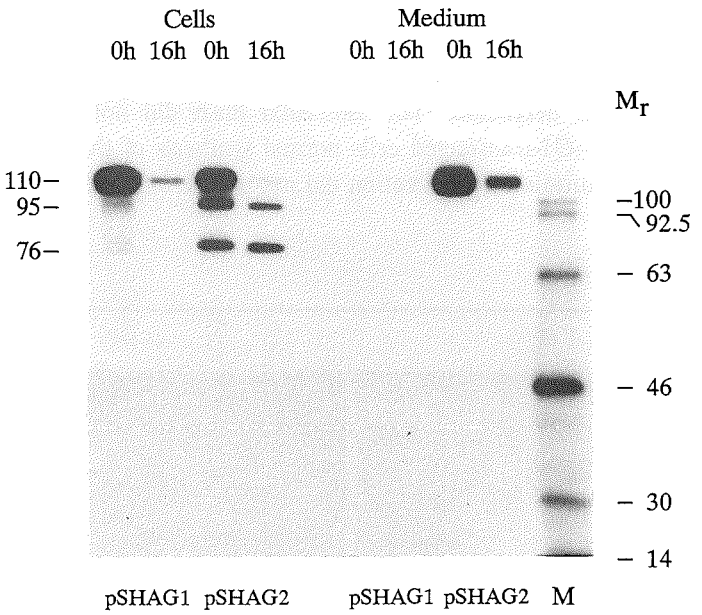

Figure 4. Processing of pSHAG1- and pSHAG2-encoded acid α-glucosidase. pSHAG1- and pSHAG2-transfected COS cells were labelled for 3 hours with [^{3}H]leucine, and harvested directly (0 h) or after an additional chase period of 16 hours (16 h). Acid α-glucosidase was immunoprecipitated from cell homogenates and culture media and separated on SDS/PAGE. In the pulse, acid α-glucosidase was precipitated from double the volume of medium compared with the chase. The relative molecular masses are indicated. ^{14}C labelled marker proteins (M) (Amersham) were used.

There was no increase of acid α-glucosidase activity in cells or medium after transfection with pSHAG1 (Table 1). The failure of pSHAG1 to code for functional enzyme was unexpected. Therefore two other cDNA constructs (pSHAG5 and pSHAG6; Figure 1) were made to search for the difference between pSHAG1 and pSHAG2. With pSHAG5 the same results were obtained as with pSHAG2, whereas PSHAG6 resembled pSHAG1 (Table 1). In this way the difference between pSHAG1 and pSHAG2 could be assigned to the region between the *StuI* and 3' *SmaI* sites (Figure 1). On sequence analysis of this region, only one nucleotide difference was found (Figure 3). It concerned a C in pSHAG1, versus a T in pSHAG2 at position 1423, resulting in an arginine instead of a tryptophan at amino acid position 402.

Biosynthesis of acid α-glucosidase

To characterize further the effect of the pSHAG1 mutation, the biosynthesis of pSHAG1- and pSHAG2-encoded acid α-glucosidase was studied in transiently transfected cells. The results of a typical pulse-chase experiment are shown in Figure 4. In pSHAG2 transfected COS cells the 110 kD precursor was the predominant form of acid α-glucosidase after a three-hour pulse period, while the 95 kD intermediate and the 76 kD mature enzyme became the major species after a subsequent chase of 16 hours. In the culture medium of pSHAG2-transfected cells only the precursor of acid α-glucosidase was detectable. The molecular mass did not change during the chase period. In pSHAG1-transfected cells normal synthesis of the 110 kD precursor was observed, but maturation and secretion did not occur.

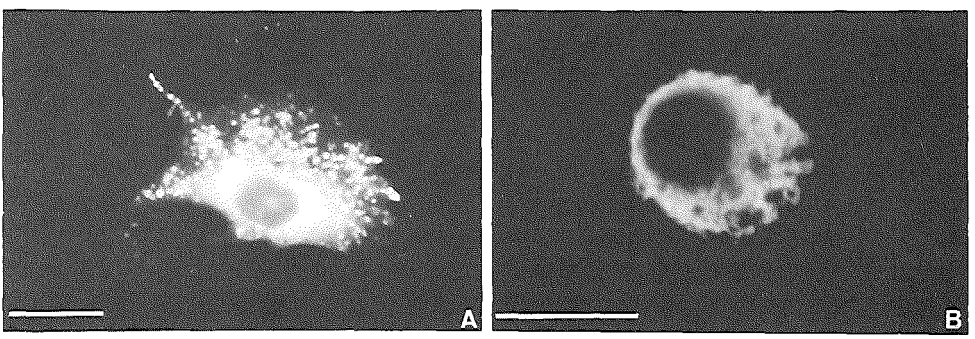

Figure 5. Localization of acid α-glucosidase in pSHAG1- and pSHAG2-transfected COS cells studied by light-microscopy. Transfected cells were incubated with rabbit antibodies against human acid α-glucosidase. Immune-complexes were visualized with goat anti-rabbit-fluorescein. A; pSHAG2 transfected cell, B; pSHAG1 transfected cell. Bars = 20 μm.

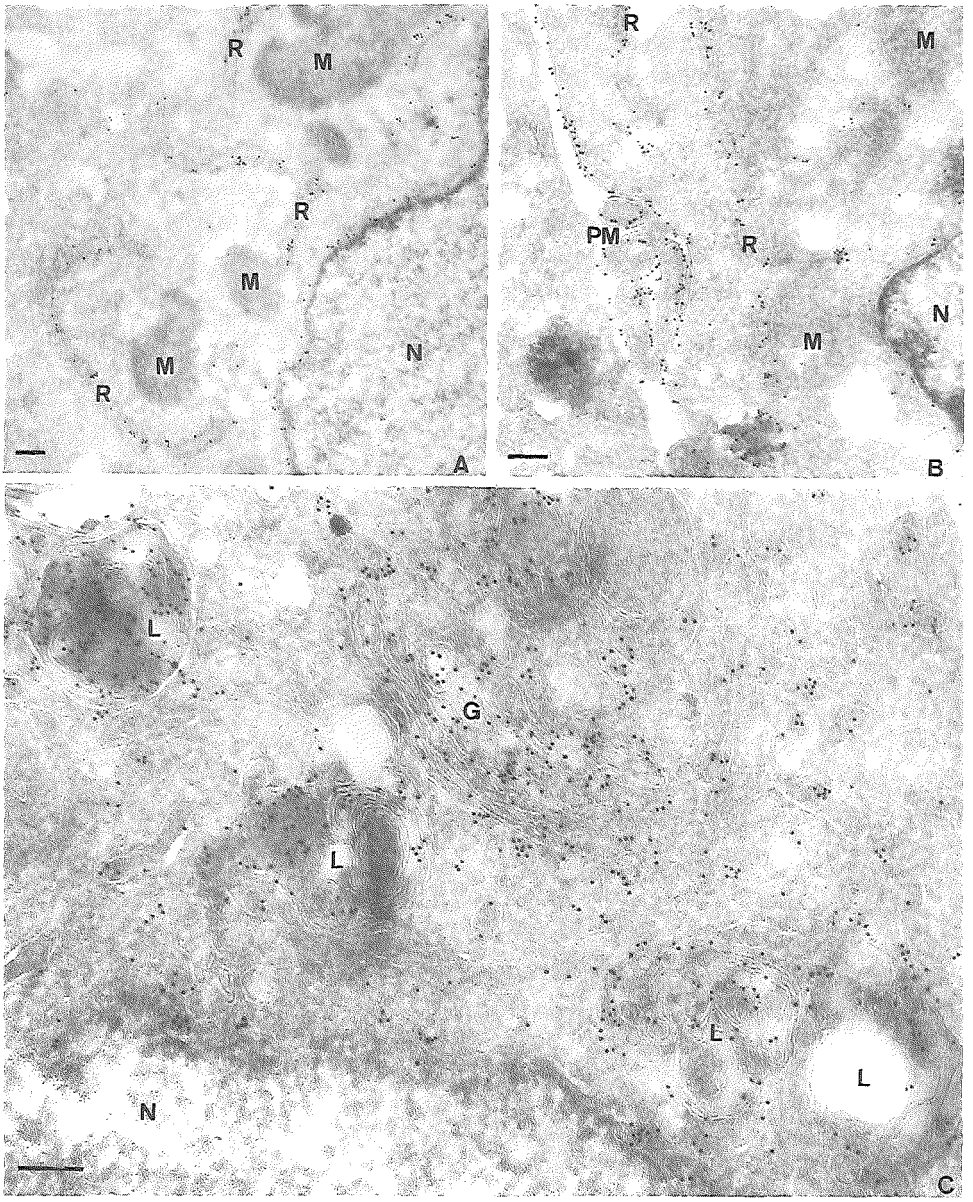

Figure 6. Localization of acid α-glucosidase in pSHAG2-transfected COS cells studied by immuno-electron microscopy. Ultra-thin cryosections were incubated with rabbit polyclonal antibodies directed against acid α-glucosidase and immune complexes were visualized with goat anti-(rabbit IgG) coupled to colloidal gold. Acid α-glucosidase was found at the nuclear envelope (A, B, C), in the endoplasmic reticulum (A, B), at the plasma membrane (B), in the Golgi complex (C) and in the lysosomes (B, C). The untransfected cell in (B) on the left did not contain any label. M, mitochondrion; N, nucleus; R, rough endoplasmic reticulum; PM, plasma membrane; L, lysosomes; G, Golgi complex. Bars = 0.2 μm.

Intracellular transport of acid α-glucosidase

The intracellular routeing of pSHAG1- and pSHAG2- encoded enzyme was compared using immunocytochemistry. Typical punctate lysosomal labelling was obtained in pSHAG2-transfected cells using immuno-light microscopy (Figure 5A). In contrast, the pattern of labelling in pSHAG1 transfected cells was that of a diffuse network spreading from the nucleus into the cytoplasm (Figure 5B). Immuno-electronmicroscopy confirmed the difference in subcellular localization. In Figure 6 the results are shown for pSHAG2. For both pSHAG1 and pSHAG2, labelling of the rough endoplasmic reticulum, including the nuclear envelope, was observed (Figure 6A-C). Also, the Golgi complex was labelled in both cases (shown in Figure 6C for pSHAG2). However, lysosomal labelling (Figure 6B and 6C) and labelling of the

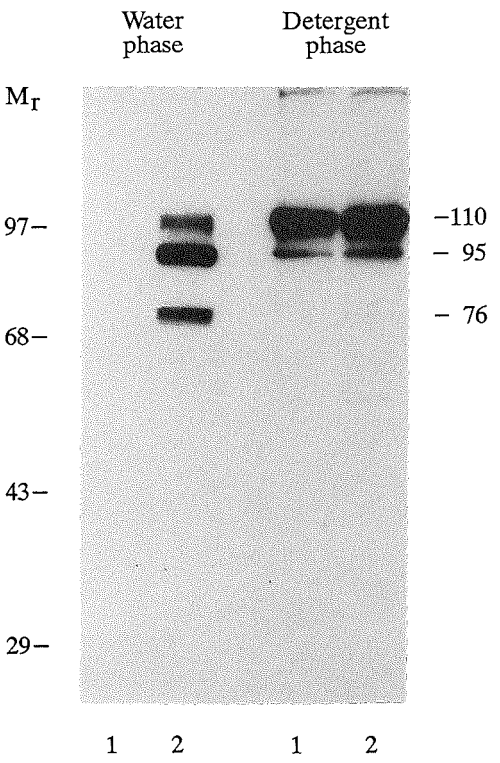

Figure 7. Triton X-114 phase separation of pSHAG1- and pSHAG2-transfected COS cells. A phase separation using Triton X-114 was performed on transfected COS cells. Acid α-glucosidase was immunoprecipitated from both the water phase and the detergent phase and analysed on SDS/PAGE followed by immunoblotting (1, pSHAG1 transfected cells; 2, pSHAG2 transfected cells). Relative molecular masses are indicated. Prestained high molecular weight protein markers (M) from BRL were used.

plasma membrane (Figure 6B) were only obtained in pSHAG2-transfected cells.

The plasma membrane localization was rather unexpected, and therefore studied in more detail. To this end, pSHAG2 transfected COS cells were harvested by scraping and washed three times in iso-osmotic buffer with or without 10 mM mannose 6-phosphate. Immuno-electron microscopy revealed that mannose 6-phosphate did not abolish membrane labelling (results not shown).

In order to investigate the possible existence of membrane-associated forms of acid α-glucosidase, transfected COS cells were homogenized and a phase separation with Triton X-114 was performed (Figure 7). The major component in the water phase of pSHAG2-transfected cells was the 95 kD processing intermediate. In addition, a soluble precursor of approximately 110 kD and the 76 kD mature form of acid α-glucosidase were present. No soluble forms of acid α-glucosidase were

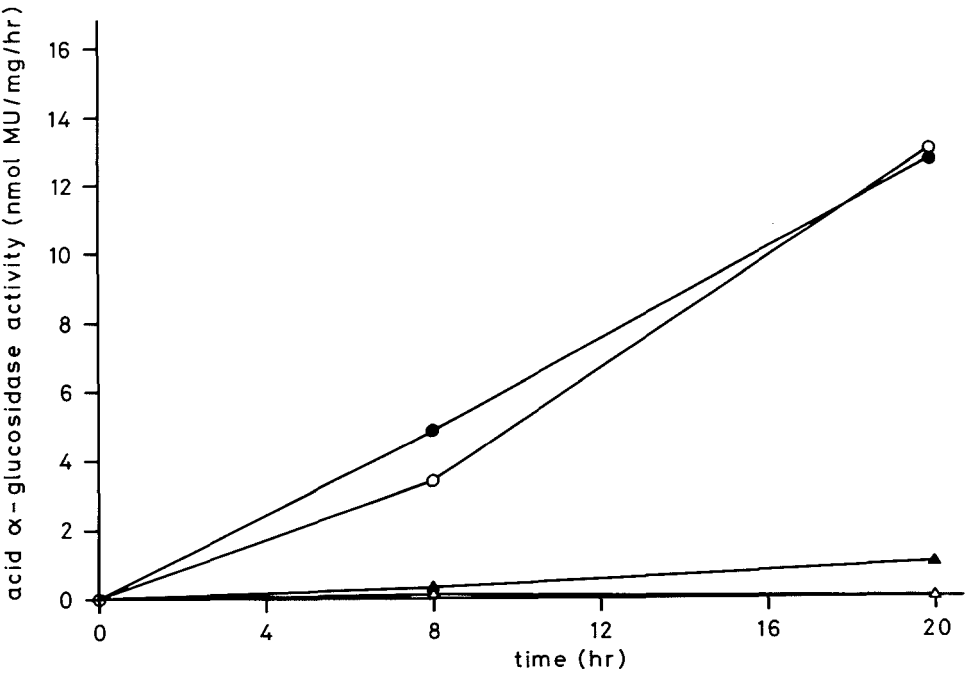

Figure 8. Uptake kinetics of acid α-glucosidase. Enzyme-deficient fibroblasts from a patient with glycogenosis type II were maintained in culture medium containing acid α-glucosidase secreted by pSHAG2-transfected COS cells (closed circles), bovine testis acid α-glucosidase (open circles), human placental acid α-glucosidase (closed triangles), or in culture medium without enzyme addition (open triangles). Equal amounts of enzyme (equivalent to 100 nmol MU/hour) were added. The intracellular acid α-glucosidase activity was measured at different time points.

precipitable from the water phase of pSHAG1-transfected cells. The detergent phase of both pSHAG1- and pSHAG2- transfected cells contained almost exclusively the 110 kD precursor (Figure 7).

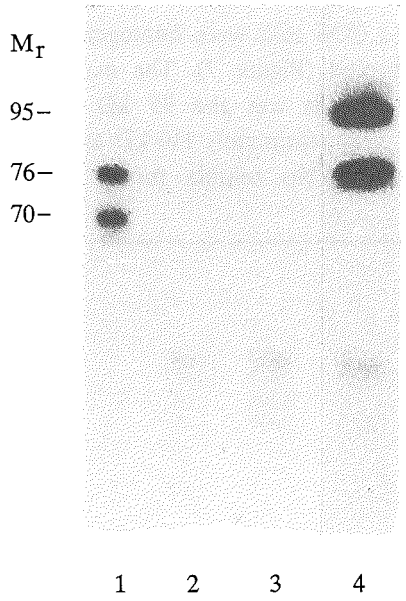

Figure 9. Processing of acid α-glucosidase after uptake by enzyme-deficient fibroblasts. Enzyme-deficient fibroblasts from a patient with glycogenosis type II were cultured in medium conditioned by untransfected COS cells (lane 2), pGA293 transfected COS cells (lane 3), or pSHAG2 transfected COS cells (lane 4). Acid α-glucosidase was immunoprecipitated from cell homogenates and separated on SDS/PAGE, followed by immuno-blotting. Acid α-glucosidase isolated from human placenta was applied as marker (lane 1).

Enzyme uptake and correction

To further analyse the structural and functional characteristics of acid α-glucosidase encoded by pSHAG2, fibroblasts and myoblasts from patients with an acid α-glucosidase deficiency were cultured in the medium of transfected COS cells. Figure 8 shows that pSHAG2-produced enzyme precursor was taken up by fibroblasts with the same efficiency as mannose 6-phosphate containing acid α-glucosidase purified from bovine testis, while the uptake of non-phosphorylated human placental enzyme was much less efficient. Moreover, the uptake of enzyme from the COS cell culture medium was inhibited by 10 mM mannose 6-phosphate (data not shown). This

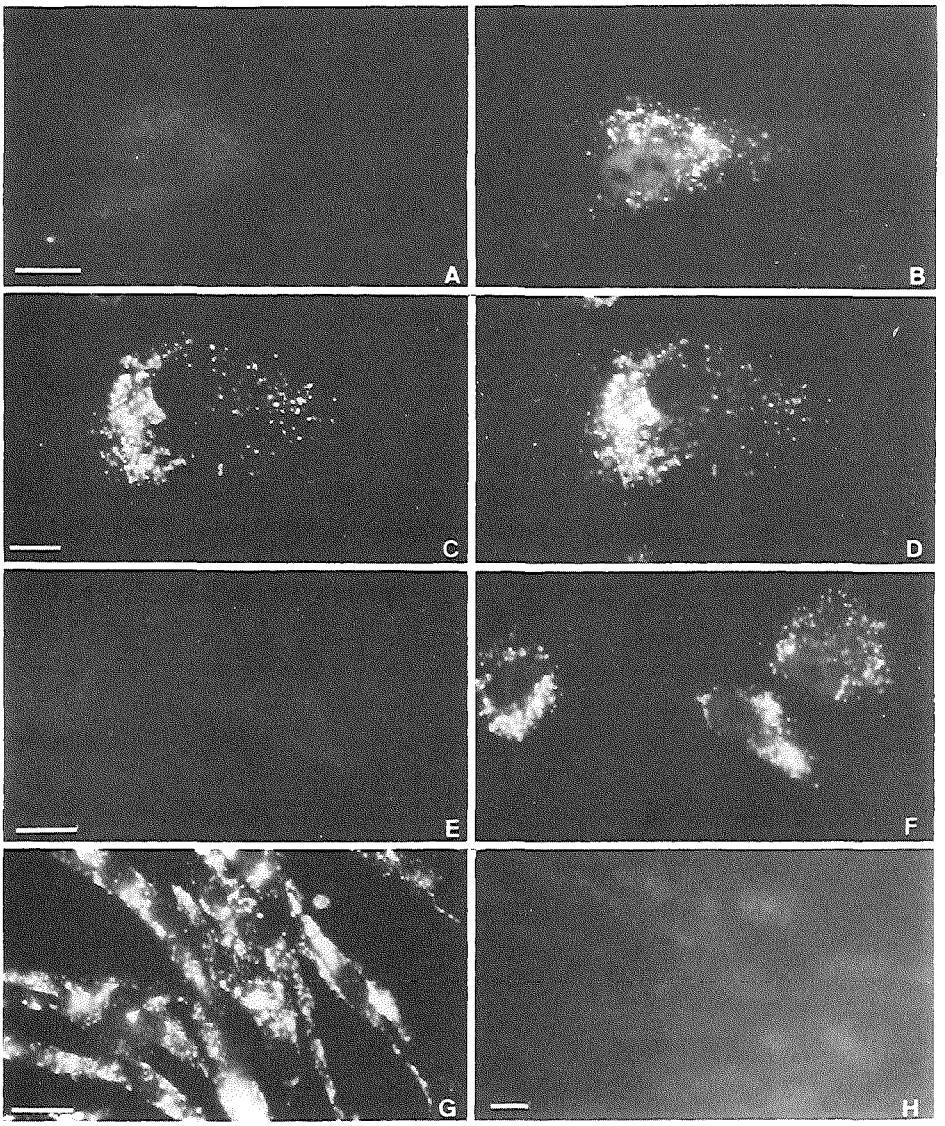

Figure 10. Intracellular localization of acid α-glucosidase studied by immuno-light microscopy. Cultured fibroblasts and muscle cells were fixed and incubated with antibodies directed against acid α-glucosidase and ß-hexosaminidase. Immune complexes were visualized with goat anti-mouse or goat anti-rabbit antibodies coupled to fluorescein and rhodamine, respectively. Panels A-F, enzyme-deficient fibroblasts from a patient with glycogenosis type II stained for acid α-glucosidase (A, C, E) and the same cells stained for N-acetyl-ß-hexosaminidase (B, D, F). Panels A and B, untreated cells; Panel C and D, cells treated with culture medium from pSHAG2-transfected COS cells; Panel E and F, cells treated with culture medium from pGA293 transfected COS cells; Panels G and H, enzyme-deficient myoblasts from a patient with glycogenosis type II treated with culture medium from pSHAG2- (G) or pGA293- (H) transfected COS cells and stained for acid α-glucosidase. Bars = 20 μm.

suggests that the secreted enzyme precursor is equipped with the mannose 6-phosphate recognition marker. After 4 days the acid α-glucosidase activity of the fibroblasts had increased from 0.9 to 47.8 nmol MU/h per mg protein, while the activity in myoblasts went up from 0 to 5.1 nmol MU/h per mg protein.

Immunoblot analysis revealed that the 110 kD acid α-glucosidase precursor secreted by COS cells was converted after internalization by fibroblasts to mature enzyme of 76 kD via the known 95 kD processing intermediate (Figure 9). Culture medium from pGA293 transfected cells served as a control.

Immuno-light microscopy was employed to establish the intracellular localization of endocytosed acid α-glucosidase. Before enzyme addition, the fibroblasts of the patient were completely devoid of acid α-glucosidase (Figure 10A), but had a normal lysosomal localization of ß-hexosaminidase (Figure 10B). Uptake of acid α-glucosidase (Figure 10C) resulted in a labelling pattern identical to that of ß-hexosaminidase (Figure 10D). Fibroblasts treated with COS/pGA293-conditioned culture medium were taken as a control and were negative for acid α-glucosidase (Figure 10E) but positive for ß-hexosaminidase (Figure 10F). A characteristic lysosomal distribution of label was also obtained in myoblasts of a glycogenosis type II patient incubated with culture medium conditioned by pSHAG2 transfected COS cells (Figure 10G). Exposure of the enzyme deficient myoblasts to culture medium from pGA293 transfected COS cells had no effect (Figure 10H). Figure 11 illustrates

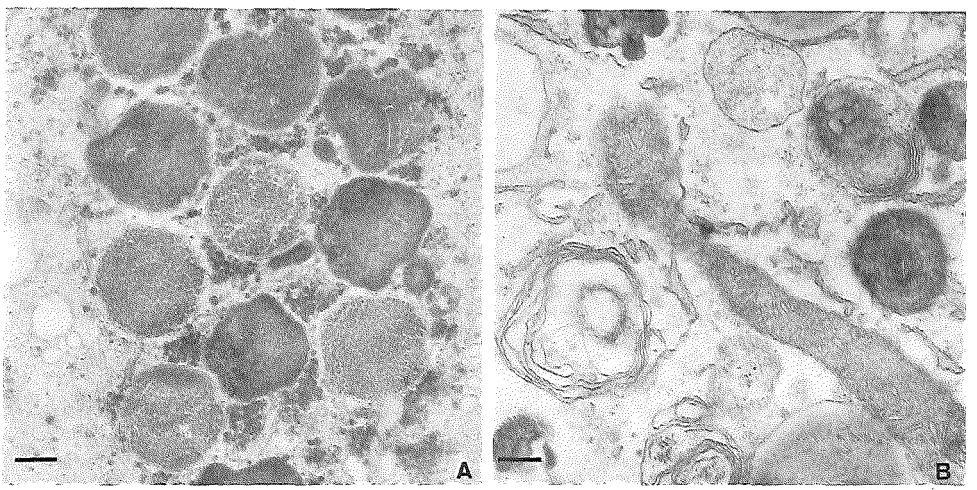

Figure 11. Lysosomal glycogen content of fibroblasts studied by transmission electronmicroscopy. A, lysosomal glycogen storage in cells from a patient with glycogenosis type II; B, lysosomes in cells from the same patient after four days of culture in medium conditioned by pSHAG2-transfected COS cells. Bars = 0.2 μm.

that enzyme uptake resulted in the degradation of accumulated lysosomal glycogen.

Discussion

Two full-length acid α-glucosidase cDNA constructs differing in only one base pair were expressed in mammalian cells. pSHAG1 represents the originally cloned cDNA (Hoefsloot et al., 1988) and codes for an arginine residue at position 402 of the amino acid sequence. pSHAG2 encoded enzyme has a tryptophan residue at this position. It is shown that the synthesis and post-translational modification of pSHAG2-encoded acid α-glucosidase in transfected cells proceeds as in human fibroblasts (Hasilik and Neufeld, 1980a; Reuser et al., 1985). A glycosylated 110 kD precursor is initially formed and then converted stepwise to the mature 76 kD enzyme. Expression of pSHAG2 leads to an increase in the acid α-glucosidase activity and secretion of a catalytically active precursor. In contrast, pSHAG1 encoded acid α-glucosidase does not mature and is not catalytically active. The combined results of pulse-chase experiments and immuno-electron microscopy suggest that the precursor is degraded after it has reached the Golgi complex. Therefore we conclude that pSHAG2 contains a wildtype cDNA sequence, whereas pSHAG1 cDNA is mutated.

The mutation in pSHAG1 may either represent a natural variant or a cloning artifact. Amino acid 402 is not obviously located near proteolytic cleavage sites used during maturation, nor near the putative active site (Hoefsloot et al., 1988). Therefore we assume that the lack of maturation and the deficiency of catalytic activity are secondary effects. The change from tryptophan to arginine is from a hydrophobic to a hydrophilic amino acid, and influences the secondary structure of acid α-glucosidase locally. Computer analysis of the secondary structure according to Chou and Fasman (1978) predicts the loss of a ß-sheet and the introduction of a turn. This mis-folding may decrease the stability of the precursor.

The difference between pSHAG1 and pSHAG2 is instructive for the intracellular routeing of acid α-glucosidase. As a consequence of the mutation, pSHAG1 encoded enzyme does not reach the lysosomes, is not secreted, and is not localized at the plasma membrane either. Thus the transport pathways to these three locations diverge after passage of the precursor through the Golgi complex.

The localization of acid α-glucosidase at the plasma membrane has not been observed previously in normal human fibroblasts, and was therefore a rather unexpected finding (Van Dongen et al., 1985; Reuser et al., 1987). It is possible that the membrane association of the acid α-glucosidase precursor (as demonstrated by Triton X-114 phase separation) is responsible for transport to the plasma membrane. This alternative route may gain significance when the enzyme is overexpressed, as in COS cells, or when a specific transport pathway to the apical membrane exists, as in polarized epithelial cells. Plasma membrane localization for acid α-glucosidase has

been demonstrated recently for the latter cell type (Fransen et al., 1988; Oude Elferink et al., 1989). Membrane association may also explain the high residual activity of acid α-glucosidase in I-cells, in which the mannose 6-phosphate receptor-dependent pathway is not functional (Van Dongen et al., 1985; Tsuji and Suzuki, 1987). In this situation acid α-glucosidase is also diverted to an extra-lysosomal compartment (Van Dongen et al., 1985).

The nature of the membrane association is still unclear. One hypothesis is that it is related to the proposed homology between acid α-glucosidase and the typical brush border enzyme sucrase-isomaltase (Hoefsloot et al., 1988). The latter enzyme complex is anchored in the apical membrane of intestinal epithelial cells via the uncleaved signal peptide (Hunziker et al., 1986; Semenza, 1989). It is conceivable that the signal peptide of acid α-glucosidase has a similar dual function, despite the presence of a potential cleavage site (Von Heijne, 1986). Another possibility is binding via a glycophospholipid membrane anchor, shown to act as an apical targeting signal in polarized epithelial cells (Lisanti et al., 1989). We consider that binding via the mannose 6-phosphate receptor less likely, since mannose 6-phosphate did not release the enzyme from the surface of transfected cells.

The experiments described in Figures 10 and 11 form the basis for the possible application of enzyme replacement therapy in glycogenosis type II. We have demonstrated in the past that lysosomal glycogen storage in cultured fibroblasts and muscle cells from patients with glycogenosis type II can be prevented or reversed by adding mannose 6-phosphate containing acid α-glucosidase to the culture medium (Van der Ploeg et al., 1988a, 1988b). In another series of experiments evidence was obtained for uptake of acid α-glucosidase in heart and skeletal muscle of mice after intravenous enzyme administration (Van der Ploeg et al., 1991). This indicates that enzyme replacement therapy for glycogenosis type II is feasible. Our present data show that the cDNA encoded acid α-glucosidase which is secreted by transfected COS cells is taken up by fibroblasts and muscle cells with the same efficiency as mannose 6-phosphate containing enzyme from bovine testis. The uptake is inhibited by mannose 6-phosphate and is apparently mediated by the mannose 6-phosphate receptor. Once internalized, the acid α-glucosidase precursor is converted to mature enzyme and lysosomal glycogen is degraded. These features make cDNA-encoded human acid α-glucosidase suitable for enzyme replacement therapy in glycogenosis type II.

Materials and methods

Construction of full length cDNA

Standard techniques were used (Sambrook et al., 1989) to construct full-length cDNA clones coding for human acid α-glucosidase in the *Eco*RI site of the eukaryotic expression vector pSG5 (Green et al., 1988). The unique *Stu*I site present in acid α-glucosidase cDNA clones (Hoefsloot et al., 1988) was used

to generate pSHAG1 and pSHAG2 (See Figure 1). pSHAG5 and pSHAG6 were constructed by exchanging the *Sma*I-fragments of pSHAG1 and pSHAG2 (Figure 1).

DNA sequence analysis

Double-stranded pSHAG1 or pSHAG2 DNA was used as a template. Oligonucleotides complementary to the cDNA were used to prime sequence reactions with the Sequenase sequencing kit according to the specifications of the manufacturer (USB, Cleveland, Ohio, U.S.A.).

RNA transcription/translation

Plasmid DNA (1 μg), linearized with *Bgl*II, was used as a template in a transcription reaction with 3 units T7 polymerase (Boehringer, Mannheim, Germany), 10 mM dithiothreitol, 1 unit RNAase inhibitor (Promega, Madison, WI, U.S.A.), 0.8 mg of BSA/ml, 500 μM ATP, 500 μM CTP, 500 μM UTP, 50 μM GTP, and 500 μM dGpppG in a buffer with 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 2 mM spermidine (Melton et al., 1984). The reaction was carried out for 60 min at 40°C, whereafter the template was digested by addition of 1 unit RNAase free DNAase (Boehringer) per μg DNA. Nucleotides were removed by Sephadex G50 filtration (spin-column), and the RNA concentration was measured. Typically, 0.2 μg of RNA was used for *in vitro* translation in a rabbit reticulocyte lysate system (Promega, Madison, U.S.A.), containing [^{35}S]methionine (spec. act. 1130 Ci/mmol, Amersham International, Bucks., U.K.) in the presence or absence of canine pancreatic microsomes (Amersham) according to the protocol provided by the manufacturer. Translations were analysed by electrophoresis on a 12% polyacrylamide gel with 1% cross-linking in the presence of SDS as described by Laemmli (1970). After fixation and drying, gels were exposed to Kodak XAR films for 1 - 3 days.

Transfection procedures

Cells were cultured routinely in Dulbecco's modified Eagle's medium, supplemented with antibiotics and 10% fetal calf serum, under air/CO_2 (9:1). Acid α-glucosidase in the serum was inactivated by incubating the serum for 4 hours at 37°C and pH 10, followed by neutralization.

Monkey kidney COS-1 cells were transfected using the DEAE-dextran method as described (Oshima et al., 1988). In short, 2.10^5 cells in 3.5 cm tissue-culture dishes were transfected with DNA, at a final concentration of 20 μg DNA/ml of culture medium, in the presence of DEAE-dextran (200 μg/ml, Mr approximately 500.000, Pharmacia, Uppsala, Sweden). After 16 hours the cells were incubated for 3 hours with 100 μM chloroquine and harvested with trypsin at different time points, or they were used for pulse labelling.

HeLa cells were transfected using a modification of the calcium-phosphate method (Graham and Van der Eb, 1973). In short, 3 μg DNA was added to 2.10^5 cells in 3.5 cm tissue-culture dishes as a CaPi-precipitate. After 16 hours the cells were treated with 30% (v/v) DMSO for 30 min and then harvested with trypsin at the desired time.

Measurement of acid α-glucosidase activity

Cell lysates were prepared by repeated freezing and thawing of cell pellets in water, and debris was pelleted by spinning for 15 min. at 10,000 g. The acid α-glucosidase activity in culture media and cell lysates was measured using the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside as described (Galjaard, 1980) and expressed as nmol 4-methylumbelliferone (MU) formed per hour per mg protein. The protein concentration of cell lysates was determined according to Lowry et al., (1951).

Pulse-labelling experiments

Cells were transfected as described above. After 48 hours the cells were pre-incubated for one hour in leucine-free medium. This medium was replaced with leucine-free medium to which [^{3}H]leucine (190 Ci/mmol; Amersham) had been added at a final concentration of 75 μCi/ml. The cells were pulse-labelled for three hours, and harvested directly or after a subsequent chase of 16 hours. Acid α-glucosidase was immunoprecipitated from cell extracts and media with a polyclonal antibody preparation against human acid α-glucosidase, and analysed by polyacrylamide gel electrophoresis in the presence of SDS as described previously (Reuser et al., 1985).

Triton X-114 phase separation

For assessment of membrane association of acid α-glucosidase, COS cells were harvested 96 hours after transfection by scraping, and washed twice in phosphate-buffered salin (10 mM sodium phosphate/150 mM NaCL, pH 7.2). Cells were solubilized in a buffer containing 10 mM Tris-HCL (pH 7.4), 150 mM NaCl, and 0.6% Triton X-114 at 0°C. Proteins were separated in hydrophilic (water phase) and membrane-associated (detergent phase) fractions as described (Bordier, 1981).
Acid α-glucosidase was immunoprecipitated from the detergent phase and from the water phase with rabbit anti-(human acid α-glucosidase) antibodies. Different molecular forms of acid α-glucosidase were separated by PAGE in the presence of SDS (Laemmli, 1970), and blotted onto nitrocellulose (Towbin et al., 1979). Acid α-glucosidase was visualized using polyclonal rabbit anti-human acid α-glucosidase antibodies in combination with ^{125}I protein A as described earlier (Reuser et al., 1987). Blots were exposed to Kodak XAR films for 1-3 days.

Immunocytochemistry

For application of lightmicroscopy, the cells were seeded in low density on cover slips on the evening before analysis, and prepared for immunocytochemistry as described previously (Van Dongen et al., 1984). Incubations were performed with mouse polyclonal antibodies against human acid α-glucosidase (De Jonge et al., 1985) and rabbit polyclonal antibodies against ß-hexosaminidase (Reuser et al., 1985). Immune complexes were visualized with goat anti-(mouse IgG) conjugated to fluorescein and goat anti-(rabbit IgG) conjugated to rhodamine.

For immuno-electron microscopy, cells were harvested by scraping and fixed in 1% acrolein and 0.4% glutaraldehyde. Cell pellets were embedded in gelatin. Ultra-thin cryosections were immunostained by incubation with rabbit polyclonal antibodies against human acid α-glucosidase, followed by an incubation with 10 nm colloidal gold coupled to goat anti-(rabbit IgG) (Jansen Pharmaceutica, Beerse, Belgium) as described previously (Van Dongen et al., 1985).

For demonstration of glycogen in transmission electronmicroscopy, cells were fixed after four weeks of confluency as described (De Bruijn, 1973) and embedded in epon.

Miscellaneous

For assessment of mannose 6-phosphate receptor binding, transfected cells were harvested by scraping and resuspended in 10 mM sodium phosphate buffer (pH 7.2) containing 150 mM sodium chloride, with or without 10 mM mannose 6-phosphate. They were washed three times in the same buffer, and prepared for immuno-electronmicroscopy as described above.

Acid α-glucosidase was isolated from bovine testis and human placenta as described before (Reuser et al., 1985).

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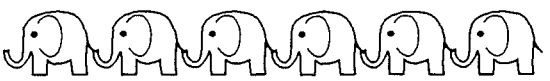
CHAPTER 6



Characterisation of the human lysosomal α-glucosidase gene[1]

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Summary

The gene coding for human lysosomal α-glucosidase was cloned, and its structure was determined. The gene is approximately 20 kb long, and contains 20 exons. The first exon is non-coding. The coding sequence of the putative catalytic site domain is interrupted in the middle by an intron of 101 bp. This intron is not conserved in the highly homologous region of the human and rabbit isomaltase genes. The promoter region was defined by a CAT-assay and the start of the mRNA was determined by primer extension. The promoter has features characteristic of a "house-keeping" gene. The GC-content is high (80%) and distinct TATA and CCAAT motifs are lacking. Two potential binding sites for the AP-2 transcription factor are present. Four potential Sp-1 binding sites are located downstream of the 5' end of the mRNA.

Introduction

Lysosomal α-glucosidase (acid α-glucosidase; glucan 1,4-α-glucosidase; EC 3.2.1.3) is essential for the degradation of lysosomal deposits of glycogen. Inherited enzyme deficiency leads to the lysosomal glycogen storage disease type II (glycogenosis type II, Pompe disease) (Hers, 1963). Several distinct abnormalities in enzyme synthesis and post-translational modification have been discovered in the various clinical phenotypes of this disease (Reuser et al., 1985, 1987; Van der Ploeg et al., 1988). The full-length cDNA coding for acid α-glucosidase has been cloned (Hoefsloot et al., 1988), and was expressed in mammalian cells (Hoefsloot et al., 1990a). The cDNA-encoded enzyme was shown to have the same characteristics as the endogenous acid α-glucosidase of human fibroblasts, with respect to intracellular transport, post-translational modification and function. One of the remarkable features of acid α-glucosidase is its sequence similarity with both subunits of the intestinal sucrase-isomaltase enzyme complex (Hoefsloot et al., 1988). Based on this similarity, the

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catalytic site of acid α-glucosidase was assigned tentatively (Quaroni & Semenza, 1976; Hunziker et al., 1986). In the present report we describe the organisation of the acid α-glucosidase gene and the characteristic features of the promoter region. The gene structures around the putative catalytic site of acid α-glucosidase and isomaltase are compared.

Results

Gene structure

Eight overlapping λ clones hybridizing with acid α-glucosidase cDNA were isolated from a human genomic library. Together, these clones span a region of more than 33 kb (Figure 1). All hybridizing sequences were contained within three contiguous *Bgl*II-fragments of 10.5, 8.5 and 14 kb, which were subcloned in the *Bam*HI site of pTZ18. A partial restriction map was constructed and fragments containing exon sequences were identified using oligo-nucleotides corresponding to various cDNA regions. All exons and flanking regions were sequenced completely.

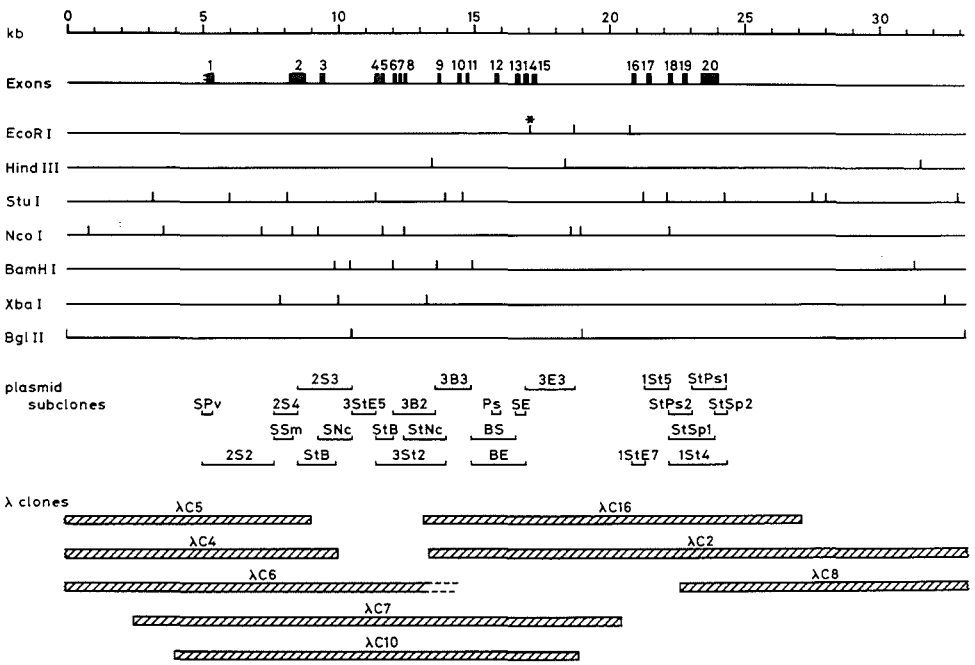

Figure 1. Organisation of the gene coding for acid α-glucosidase. A partial restriction map is given. The isolated phage clones and the plasmid subclones used for sequence analysis are indicated. The polymorphic EcoRI site is marked with an asterisk. The exons are represented by black boxes.

Table 1. Nucleotide sequence of the exon-intron boundaries

Exon	Exon size	cDNA position of exon	5' Splice donor	3' Splice acceptor	Intron size	Codon phase
1	>187	-187	CGGgtagag	tctcccgcagGCC	2800	
2	578	188-765	ACGgtgggc	tctcttctagATC	600	0
3	146	766-911	GCTgtgagt	tgtcccgcagGCT	1850	II
4	166	912-1077	ACGgtacag	gcatgtccagCCC	84	0
5	97	1078-1174	TGGgtaagc	tcccttccagATG	350	I
6	120	1175-1294	TGGgtaggg	tggcctgcagGAT	80	I
7	119	1295-1413	CTGgtgagt	tgtgctgcagGAC	88	0
8	132	1414-1545	GTGgtgtgt	ctcttcccagGAT	1120	0
9	111	1546-1656	AAGgtaggg	cgttgtccagGTA	670	0
10	114	1657-1770	ATTgtaagt	tctcttgcagGAC	101	0
11	85	1771-1855	CTGgtcagc	cctcttccagGGG	820	I
12	118	1856-1973	CAGgtgagg	accaccccagGGC	600	II
13	134	1974-2107	CAGgtaagc	gccctcccagAAA	139	I
14	152	2108-2259	CTGgtaggg	tgccctgcagCCC	190	0
15	149	2260-2408	GGAgtgagt	cccctttgcagGTT	3600	II
16	142	2409-2550	ACGgtgagt	ctcccctccagGTG	400	0
17	150	2551-2700	CAGgtacct	cccttttccagGGC	650	0
18	165	2701-2865	AATgtgagt	ctcggcccagAAC	350	0
19	153	2866-3018	AAGgcaaga	ctctttccagGTC	550	0
20	606	3019-3624				
		Consensus:	$^{A}_{C}$AGgt$^{a}_{g}$aga	$(t)_{c/n}n^{c}_{t}$agG		

Exon and intron sizes are given in numbers of base pairs. The introns 4, 6, 7, 10 and 13 were sequenced, and the exact size is indicated. The size of the other introns is based on the restriction map. Exon sequences are in upper case letter, intron sequences are in lower case. Numbers of cDNA position refer to the cDNA sequence as deposited in the EMBL/Genbank/DDBJ Nucleotide Sequence Databases under accession number Y00839. Codon phase 0 interrupts the coding sequence between two codons, phase I after the first nucleotide and phase II after the second nucleotide of a triplet.

The intron-exon boundaries were established by comparing the cDNA and genomic sequences. Following this strategy the spatial distribution of the exons and introns of the acid α-glucosidase gene was obtained (Figure 1). The gene contains 20 exons. The

start codon of acid α-glucosidase is localized near the 5' end of exon 2. Therefore exon 1 is noncoding. The stop codon is situated near the 5' end of exon 20. All intron-exon boundaries conform to the "GT/AG" rule, except for the splice donor site of exon 19, which includes a GC instead of a GT (Table 1). All three codon phases were encountered at the intron-exon boundaries.

Catalytic site domain

Based on the sequence similarity between acid α-glucosidase and isomaltase, the aspartic acid encoded by nucleotides 1771-1773 was predicted to be the essential residue in the catalytic site of acid α-glucosidase (Hoefsloot et al., 1988). Table 1 shows that an intron of 101 bp is localized between position 1770 and 1771 of the cDNA sequence, thus interrupting the coding sequence of the putative active site domain. To investigate the conservation of this intron during evolution, the corresponding domain of rabbit and human isomaltase was analysed using the

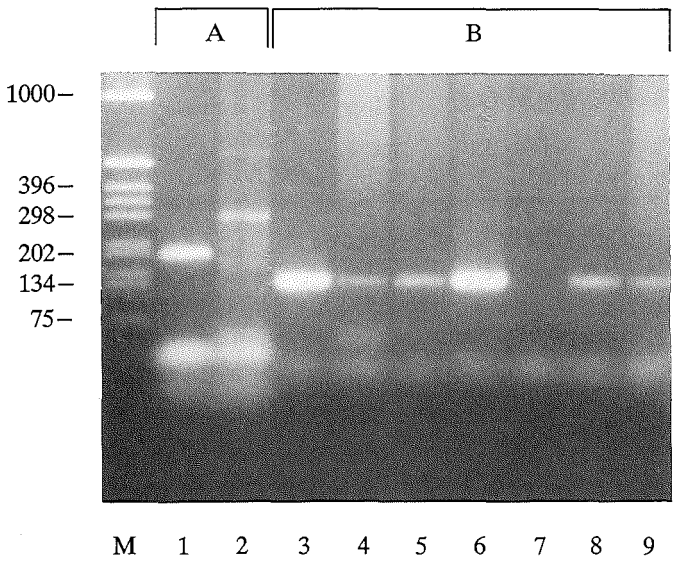

Figure 2. PCR analysis of acid α-glucosidase and isomaltase gene structure around the catalytic site. Panel A, PCR with acid α-glucosidase specific primers. These were 5'-TATGGCCCGGGTCCACTGCC (sense) and 5'-CAGGCACGTAGGGTGGGTTCTC (anti-sense). Panel B: PCR with isomaltase (human and rabbit) specific primers. These had the sequence 5'-TGATTTCACTAATCCAAACTGCA (sense) and 5'-CATTACATCCTTTTGTTGAACCT (antisense). Templates were human acid α-glucosidase cDNA (lane 1 and 7), human genomic DNAs (lane 2, 4 and 5), human isomaltase cDNA (lane 3 and 6) (Green et al., 1987), rabbit sucrase-isomaltase cDNA (lane 8) (Hunziker et al., 1986) and rabbit liver DNA (lane 9). M, marker. Fragment lengths are given in base pairs.

polymerase chain reaction. One set of primers specific for acid α-glucosidase was chosen in exon 10 and 11. Using these primers for amplification of cDNA the expected fragment of 190 bp was obtained (Figure 2). Amplification of genomic DNA

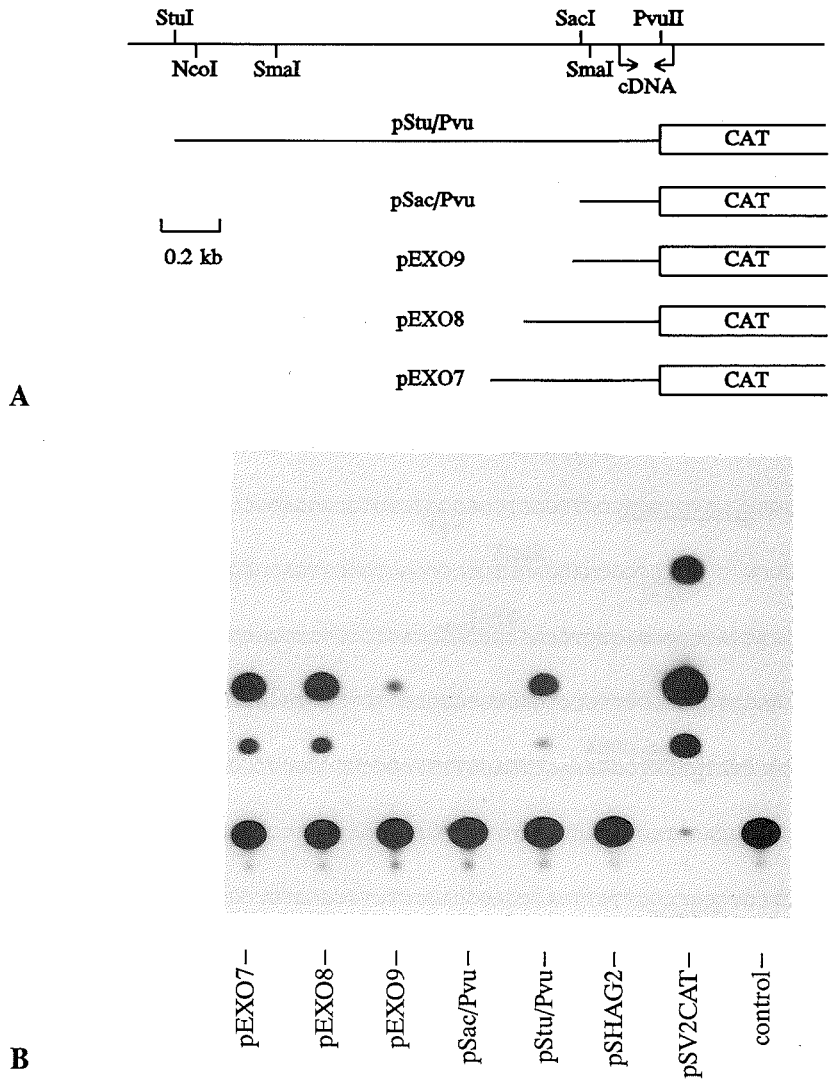

Figure 3. CAT constructs and their expression in COS-1 cells. A. Restriction map of the 5' end of the acid α-glucosidase gene. The various constructs used in the CAT assay are indicated. The arrows in the top line indicate the beginning of the longest cloned cDNA and the end of exon 1. B. CAT assays with lysates from COS-1 cells transfected with CAT constructs. Mock-transfected cells serve as a control. pSV2CAT, SV40 promoter in front of the CAT gene.

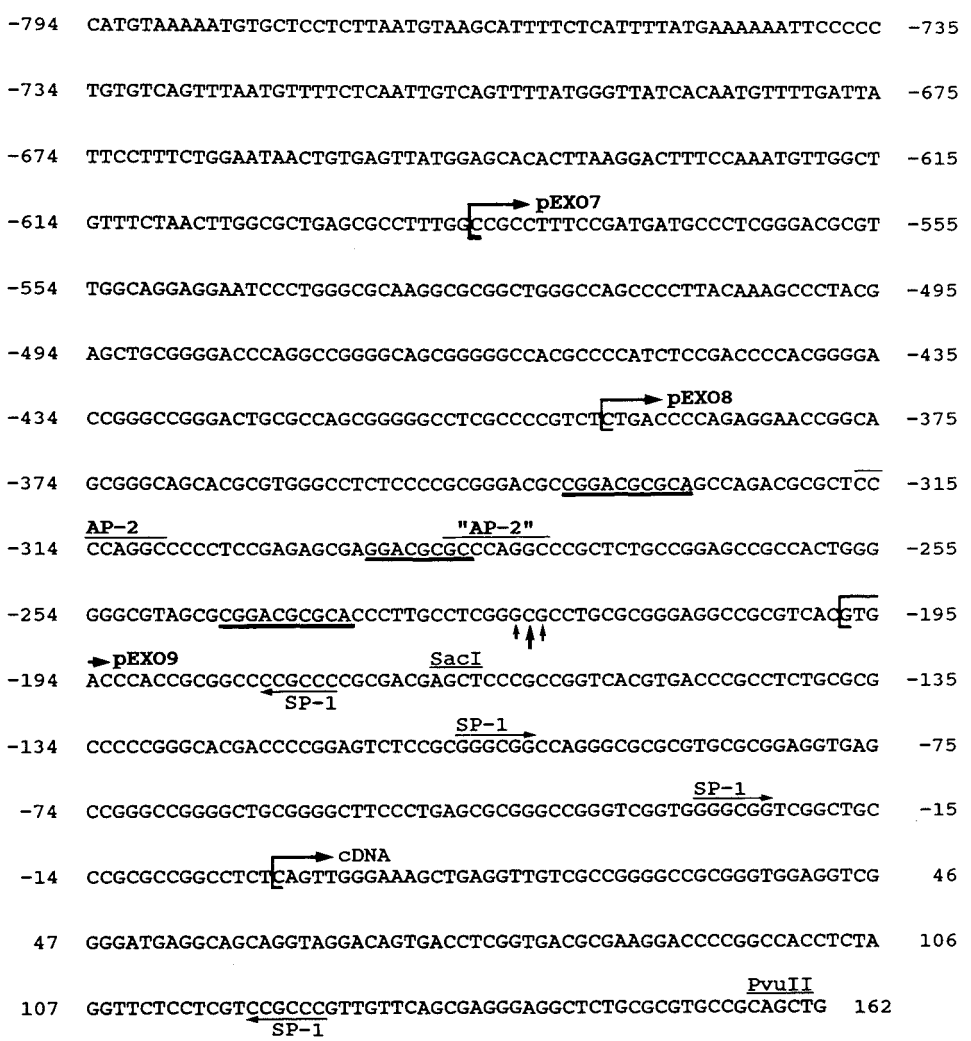

Figure 4. Sequence of the 5' end of the acid α-glucosidase gene. The 5' end of the exonuclease III-generated clones and the beginning of the longest cloned cDNA are indicated, as well as some restriction sites. Thick line, 10(8) bp repeat; AP-2, putative binding sites for the AP-2 transcription factor; SP-1, putative binding sites for the SP-1 transcription factor. Arrows indicate the transcription initiation site.

with the same primers resulted in the expected longer fragment. A second set of primers was chosen to analyze the corresponding domain in rabbit and human isomaltase using the published cDNA sequences (Hunziker et al., 1986; Green et al., 1987). The amplified cDNA and genomic fragments of isomaltase have exactly the same size. Thus the sequence coding for the catalytic site of human and rabbit isomaltase is not interrupted by an intron.

Promoter region and transcription initiation site

To define the promoter region, two genomic fragments of different length were subcloned in front of the bacterial CAT gene (Figure 3). The longer 2 kb fragment (*Stu*I/*Pvu*II) did promote CAT activity in transfected COS cells. No activity was detected with a construct containing the smaller 325 bp fragment (*Sac*I/*Pvu*II). To determine the position of the promoter region more precisely, the 2 kb fragment was shortened from the 5' end on using exonuclease III (Figure 3). Transient expression of these constructs in COS cells showed that only the shortest construct (pEXO9) has lost promoter activity. The other constructs were equally effective in expressing CAT activity. Thus, the promoter region does not extend upstream of clone pEXO8. The nucleotide sequence comprising the 5' end of the acid α-glucosidase gene is given in Figure 4. The startpoints of the exonuclease-generated clones, as well as the beginning of the longest cloned cDNA are indicated.

The 5' end of the acid α-glucosidase mRNA was determined by primer extension of a 28-mer complementary to position -71 to -98 (Figure 4). Using this oligonucleotide the longest fragment obtained had a length of 150-152 nucleotides (Figure 5). This places the transcription initiation site of acid α-glucosidase between position -220 and -222. In addition, a smaller fragment of 134 nucleotides was detected, which could be explained by premature termination caused by secondary structures. Smaller fragments than expected were also obtained using RNA from this region synthesized *in vitro* (results not shown).

The promoter region defined by CAT assay and primer extension does not contain a typical CCAAT box or a TATA-resembling motif (Figure 4). A potential AP-2 binding site with a perfect match to the consensus sequence (Mitchell & Tjian, 1989) is located at position -316 to -309, and a second site with one mismatch is located at position -287 to -280. There are several direct repeats, the longest of which is found at position -338 to -329 and -244 to -235. The middle eight base pairs of this repeat recur at position -293 to -286. The sequence (Figure 4) includes four potential Sp-1 binding sites (Dynan, 1986; Mitchell & Tjian, 1989), two in sense and two in anti-sense orientation. However, these are all located in the untranslated region of the acid α-glucosidase mRNA. The G+C content is 80% and the observed / expected ratio of the CpG dinucleotide is 0.9. The combined features are typical for the promoter of a "housekeeping" gene (Dynan, 1986).

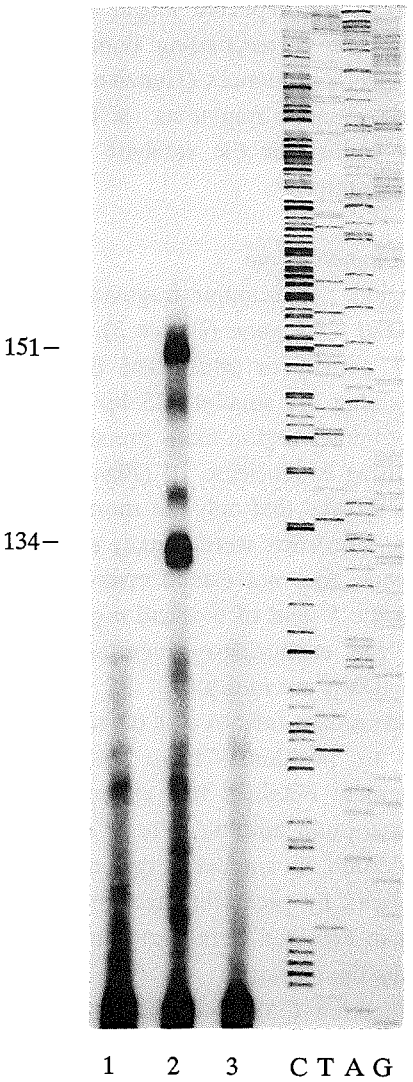

Figure 5. Primer extension with an oligonucleotide complementary to position -71 to -98. A sequence reaction from a fragment of the 5' region was used as a size marker. Lane 1, primer only; lane 2, primer extension with 100 μg total RNA isolated from human fibroblasts; lane 3, reaction with 100 μg tRNA. Numbers on the left indicate length of fragments in nucleotides.

DNA polymorphisms

Sequence comparison of all exonic DNA sequences and the previously published acid α-glucosidase cDNA revealed several differences. Some of these differences

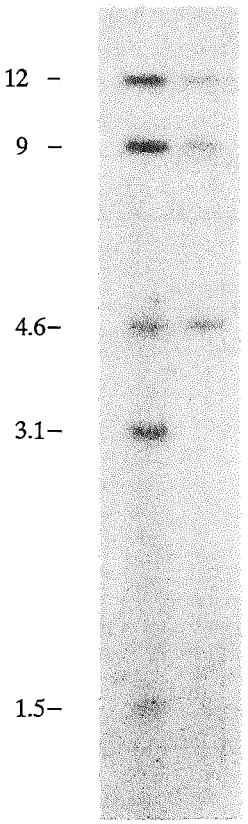

Figure 6. EcoRI polymorphism in the acid α-glucosidase gene. HindIII/EcoRI double-digested DNA was analysed by Southern blotting using the full-length acid α-glucosidase cDNA as a probe. The length of hybridizing fragments is indicated in kilobases.

appeared to be artificial and were found to be caused by misinterpretation of the cDNA sequence data. Others were identified as single base pair polymorphisms. These are listed in Table 2. The corrected cDNA sequence has been submitted to the EMBL/Genbank/DDBJ Nucleotide Sequence Databases (accession number Y00839).

According to the restriction map in Figure 1, three genomic *Eco*RI fragments are expected to hybridize with acid α-glucosidase cDNA. The 1.5 kb fragment, however, containing exon 15, was not detected in previous Southern blot hybridizations (Hoefsloot et al., 1988). To investigate whether the 5' *Eco*RI site of the 1.5 kb fragment (marked with an asterisk) is polymorphic, DNA of 11 unrelated individuals was analysed. To facilitate the interpretation of the results, the DNA was double

digested with *Hin*dIII and *Eco*RI. In case the *Eco*RI site is present, the 4.6 kb *Hin*dIII fragment (Figure 1) is cut in two smaller fragments of 3.1 and 1.5 kb. An example is given in Figure 6. Heterozygosity for the *Eco*RI polymorphism (Figure 6, left lane) was detected in three out of eleven cases. Sequence analysis of both alleles showed that the polymorphism is based on the variable presence of a thymidine residue in the GAA(T)TC *Eco*RI recognition sequence.

Table 2. DNA polymorphisms

cDNA position	cDNA	Genomic	Amino acid alteration
543	C	T	—
815	G	A	Arg→His (conservative)
887	A	G	His→Arg (conservative)
1423	C	T	Arg→Trp
1800	A	G	—
2772	A	G	—
3217/3218	—	G	Non-coding
3305	G	C	Non-coding
3496	T	G	Non-coding

cDNA position refers to the numbering of the cDNA sequence as deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession number Y00839

Discussion

The gene coding for human acid α-glucosidase contains 20 exons and 19 introns spread over a distance of 20 kb. The sizes of the exons and introns are not unusual for eukaryotic genes (Hawkins, 1988). The first intron is located within the 5' untranslated region, and the first exon is therefore non-coding. The ATG start codon is located 33 bp from the beginning of the second exon. The untranslated part of the first coding exon of vertebrate genes is generally short, and rarely exceeds 40 nucleotides (Hawkins, 1988). It has been suggested that introns demarcate structural and/or functional domains of proteins (Gilbert, 1985). For instance, a correlation between structural domains and intron-exon organisation was postulated for lysosomal acid phosphatase (Geier et al., 1989). Furthermore, the proteolytic cleavage site used in the maturation of the α-chain of lysosomal hexosaminidase is located at the beginning of an exon (Proia, 1988). Little information is available yet on the structural domains of acid α-glucosidase. However, the signal peptide, the pro-sequence of the acid α-glucosidase precursor and the first 61 amino acids of the 76 kD mature enzyme are all encoded in the same exon (exon 2). It is also notable that

the coding information for the putative catalytic site domain of acid α-glucosidase is interrupted by an intron. Considering the strong sequence similarity between acid α-glucosidase and isomaltase, it is surprising that no intron is present at the same site in the human and rabbit isomaltase gene.

All splice junctions conform to the "GT/AG" rule, except for the splice donor site of exon 19, having GC instead of GT. Such a splice donor site is very rare, but has been described for human and rodent APRT genes (Broderick et al., 1987) duck (Erbil & Niessing, 1983) and chicken (Dodgson & Engel, 1983) α-globin genes, and the mouse αA-crystallin gene (King & Piatigorsky, 1983).

The transcription initiation site was determined by primer extension, and was found to be located approximately 220 bp upstream from the longest cloned cDNA. This is 440 bp in front of the ATG start codon. The transcription initiation site is properly positioned within the limits of the promoter region as determined by the various constructs used in the CAT-assay. The *Sac*I/*Pvu*II fragment located 3' of the transcription initiation site lacks promoter activity. A genomic fragment starting 175 bp upstream of the transcription initiation site (clone pEXO8) has full promoter activity. The characteristics of this region are typical for the promoter of a "housekeeping" gene. The G+C content is high (80%) and the CpG dinucleotide is not depleted, meeting the requirements for a CpG-island (Gardiner-Garden & Frommer, 1987). Sequences resembling TATA motifs are absent. The CCACT sequence at position -262 to -258 is located too close to the proposed transcription initiation site to function as a CCAAT box (Breathnach & Chambon, 1981). The promoter region of a few other lysosomal enzyme genes have been studied (Proia & Soravia, 1987; Bishop et al., 1988; Neote et al., 1988; Geier et al., 1989) and all except one (glucocerebrosidase; see Horowitz et al., 1989) seem to have a promoter characteristic of a "housekeeping" gene. The presence of one, possibly two putative AP-2 binding sites (Mitchell & Tjian, 1989) in the promoter region of acid α-glucosidase is remarkable, since the AP-2 transcription factor confers inducibility of gene expression by cAMP and phorbol esters (Imagawa et al., 1987). Whether the AP-2 binding sites are relevant for acid α-glucosidase expression remains to be determined. In the 5' flanking sequences of the hexosaminidase ß-gene (Neote et al., 1988) and the α-galactosidase gene (Bishop et al., 1988), two and one putative AP-1 binding sites were found respectively. The AP-1 transcription factor confers inducibility by phorbol ester.

Several polymorphisms were found. Most were silent or conservative (Table 2). The only non-conservative difference concerns a C to T transition at nucleotide position 1423, leading to a substitution of arginine by tryptophane. Tryptophan-containing acid α-glucosidase was found to be transported to the lysosomes and to be catalytically active. The arginine-containing enzyme, however, did not mature, and was detected only in the endoplasmic reticulum and the Golgi complex, in a catalytically

inactive form (Hoefsloot et al., 1990a). The polymorphic *Eco*RI-site is situated in intron 14. The recently reported XbaI polymorphism (Hoefsloot et al., in press b) is due to the variable presence of an XbaI site in the XbaI fragment containing exons 2 and 3 (Figure 1). Both restriction fragment length polymorphisms can be used for diagnostic purposes.

Materials and methods

Isolation of genomic clones

A human genomic EMBL-3 library (CML-0, De Klein et al., 1986) was screened with a full-length human acid α-glucosidase cDNA, clone pSHAG2 (Hoefsloot et al., 1990a). Hybridizing restriction fragments of the isolated phage clones were subcloned in appropriate sites of either pTZ18 or M13mp18/mp19 (Pharmacia Biotechnology International AB, Uppsala, Sweden). The inserts were sequenced using the T7 polymerase sequencing kit according to the instructions of the manufacturer (Pharmacia Biotechnology International AB, Uppsala, Sweden). The M13 universal primer, or primers complementary to the cDNA were used.

Southern blotting

DNA was isolated from 10 ml blood obtained from unrelated Caucasians, using the high-salt extraction procedure (Miller et al., 1988). Restriction enzyme digests were performed on 10-15 μg of DNA in the appropriate buffers. DNA fragments were separated on 0.8% (w/v) agarose gels and subsequently blotted onto nitrocellulose filters. Filters were hybridized with acid α-glucosidase cDNA using standard protocols (Sambrook et al., 1989).

Polymerase chain reaction

DNA isolated from human control fibroblasts and rabbit liver was used as a template in a reaction mixture containing 100 pmol of each primer, 2 units of Amplitaq (Cetus), 50 mM Tris/HCl (pH 8.3), 3.0 mM $MgCl_2$, 25 mM KCl, 200 μg/μl BSA, 10% (v/v) dimethylsulfoxide, 5 mM ß-mercaptoethanol, 17 mM $(NH_4)_2SO_4$ and 0.1 mM of each dNTP. DNA fragments were amplified in 25 cycles (2 min of denaturation at 94 °C, 1.5 min of annealing at 57 °C, and 3 min of extension at 72 °C) using a Cetus DNA amplifier (Cetus corporation, Emeryville, CA, U.S.A.). One-third of each reaction was analysed on a 2% (w/v) Nusieve/agarose gel.

CAT assay

The TK-promoter of vector pBLCAT3 (Luckow & Schütz, 1987) was removed by digestion with *Bam*HI and *Bgl*II. Fragments of the 5' region of the acid α-glucosidase gene were cloned in this vector as follows. A 2 kb *Stu*I/*Pvu*II fragment and a 325 bp *Sac*I/*Pvu*II fragment were subcloned, respectively, in the *Sma*I site and the *Sac*I/*Sma*I sites of pSP72 (Pharmacia). Using the *Bam*HI and *Bgl*II site on either side, the insert was retrieved from this vector and cloned in the corresponding sites of pBLCAT3 in sense orientation. A series of 5' deletion clones derived from the 2 kb fragment in pBLCAT3 was generated by exonuclease III digestion. COS-1 cells were transfected with the CAT-constructs as described before (Hoefsloot et al., 1990a). Cells were harvested 72 hours after transfection and lysed by repeated freeze-thawing in 0.25 M Tris/HCl (pH 7.8). A 10.000 g supernatant was prepared and endogenous acetylases were inactivated by incubation for 10 min at 60 °C. CAT activity was determined according to Gorman et al. (1982).

Sequence analysis of promoter region

Several restriction fragments derived from the 5' end of the acid α-glucosidase gene were subcloned in M13mp18/mp19 and sequenced in both directions. In addition, relevant exonuclease III-generated CAT constructs were sequenced from their 5' end using double stranded plasmid DNA and the M13 universal primer.

Primer extension

RNA was isolated from human fibroblasts following the method of Schreiber et al. (1989). Synthetic RNA was made as described previously(Melton et al., 1984). oligonucleotides were end-labeled using [γ-^{32}P]dATP and polynucleotide kinase. Radiolabeled oligonucleotide (10^5 c.p.m.) was hybridized for 8-12 hours at 32 °C to 100 μg of RNA in a 30 μl reaction mixture containing 40 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA (pH 8.0) and 80% (v/v) formamide. The extension reaction was carried out according to Sambrook et al. (1989) and products were analysed on a 10% polyacrylamide gel with 1% cross-linking.

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CHAPTER 7

Adult and infantile glycogenosis type II in one family, explained by allelic diversity[1]

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Summary

To define the cause of clinical heterogeneity in glycogenosis type II we have studied the inheritance and molecular nature of acid α-glucosidase deficiency in a rare family with severe infantile as well as mild late-onset variants of this disease. The (mutant) acid α-glucosidase alleles of crucial family members were segregated in human-mouse somatic cell hybrids to investigate their individual function. Two types of mutant alleles were identified. The first leads to complete deficiency of acid α-glucosidase. Homozygosity of this allele is demonstrated in three cases of severe infantile glycogenosis type II in the family under study. The second mutant allele is characterized by a reduced net production of catalytically active acid α-glucosidase, resulting in partial enzyme deficiency. The eldest patient in the family, with very mild clinical symptoms, is shown to be a compound heterozygote having both types of mutant alleles. These studies emphasize the effect of allelic diversity on the level of residual acid α-glucosidase activity and the clinical course of glycogenosis type II.

Introduction

Glycogenosis type II (Pompe disease) is one of the more than 30 lysosomal storage disorders presently known (Hers 1963; Callahan and Lowden 1981; McKusick 1988; Scriver et al., 1989). The disease is caused by inherited deficiency of acid α-glucosidase (EC 3.2.1.3), the enzyme responsible for lysosomal degradation of glycogen. Rapidly progressive damage of heart and skeletal muscles is characteristic of the most severe clinical subtype and leads to death in infancy. In other forms of glycogenosis type II the first symptoms do not appear before childhood or adulthood, and pathological findings are restricted to skeletal muscle (Hers et al., 1989).

The occurrence of clinical heterogeneity is certainly not unique for glycogenosis

[1]Adapted from Am. J. Hum. Genet. **46**:45-52 (1990)

type II but is a characteristic feature of most lysosomal storage disorders (Hers and Van Hoof 1973; Reuser 1984; Reuser et al., 1988). Despite the rapidly accumulating knowledge about the specific mutations causing lysosomal enzyme deficiencies, it remains a difficult task to correlate genotype and phenotype in an attempt to explain the origin of clinical diversity. Also the cause of clinical heterogeneity in glycogenosis type II is not fully understood, but allelic variation at the acid α-glucosidase locus is presumably the most important factor (Reuser et al., 1987). Indications for the occurrence of different mutant alleles in this disease were obtained by complementation studies and by molecular analysis of acid α-glucosidase in fibroblasts, muscle cells and muscle biopsies of clinical variants (Mehler and DiMauro 1977; Reuser et al., 1978; Beratis et al., 1983; Reuser 1984; Reuser et al., 1985; Shanske et al., 1986; Reuser et al., 1987; Van der Ploeg et al., 1988; Van der Ploeg et al., 1989a). On the other hand, epigenetic factors and "genetic background" were suggested as determining the clinical phenotype to a significant extent (Angelini and Engel 1972; Ninomiya et al., 1984). In this respect, one Dutch family has attracted particular attention, with three severely affected sibs in the third generation and one patient with a very mild form of glycogenosis type II in the first (Koster et al., 1978; Busch et al., 1979; Loonen et al., 1981a; Martiniuk and Hirschhorn 1981; Ninomiya et al., 1984; Van der Ploeg et al., 1989b). Danon et al. (1986) reported on a similar interesting family.

To resolve the important issue concerning the origin of clinical heterogeneity, we analyzed the molecular and functional characteristics of the enzyme products of the different acid α-glucosidase alleles in the Dutch family.

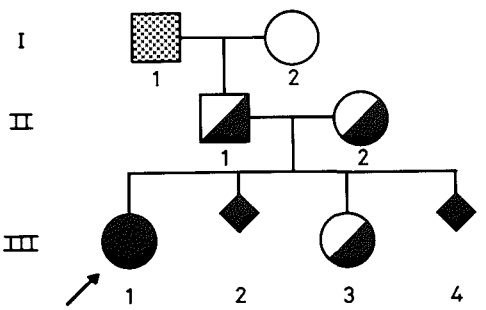

Figure 1. Pedigree of family S. Stippled gray symbol denotes adult glycogenosis type II; semiblackened symbols denote carriers; solid black symbols denote infantile glycogenosis type II. Arrow indicates index patient.

Results and Discussion

Segregation of human acid α-glucosidase alleles

Fibroblasts from I.1, II.1, II.2 (Figure 1), and a healthy control were fused with TK-deficient mouse L-cells. The resulting human-mouse somatic cell hybrids tend to loose human chromosomes spontaneously, but culturing the cells in HAT-medium provides a condition for selective retention of human chromosome 17 carrying the TK locus (Littlefield 1964). Since the gene loci for acid α-glucosidase and TK are closely linked (Nickel et al., 1982; Sandison et al., 1982; De Jonge et al., 1985; Martiniuk et al., 1985) this procedure can be used for the isolation of somatic cell hybrids containing only one of the two human acid α-glucosidase alleles (evidence is presented below).

Analysis of acid α-glucosidase activity in human-mouse somatic cell hybrids

Human-mouse hybrid clones of each individual were isolated at random, and the lysosomal acid α-glucosidase activity was measured. Human and mouse activities were distinguished using an antiserum, raised in mice, against human acid α-glucosidase. This antiserum precipitates more than 95% of human enzyme, whereas less than 1% of mouse acid α-glucosidase is bound (De Jonge et al., 1985). The results are

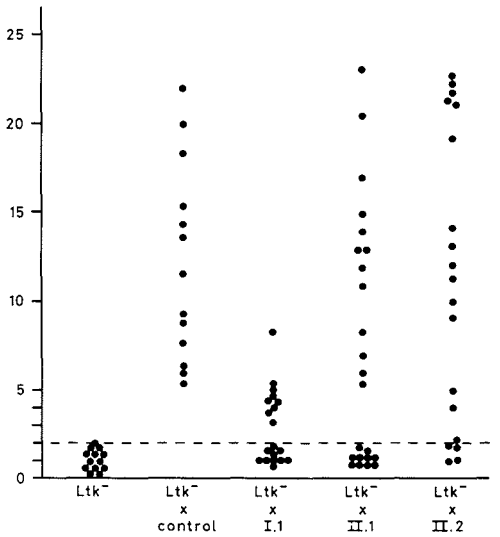

Figure 2. Acid α-glucosidase activity in LTK⁻ cells and human-mouse somatic cell hybrids. Each dot represents the acid α-glucosidase activity of a single clone measured at least in duplicate. The average acid α-glucosidase activity in LTK⁻ is set at 1.

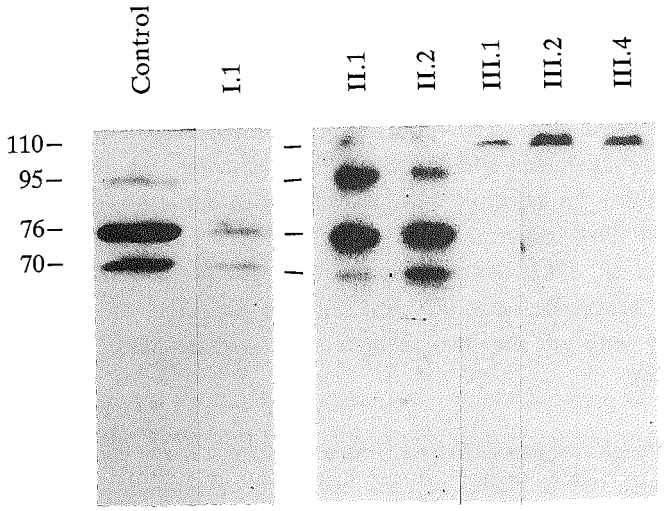

Figure 3. Molecular forms of acid α-glucosidase in fibroblasts of a control and members of family S. Proteins were separated by PAGE and blotted onto nitrocellulose filters. Acid α-glucosidase was visualized with rabbit antibodies against human placental acid α-glucosidase, in combination with ^{125}I protein A.

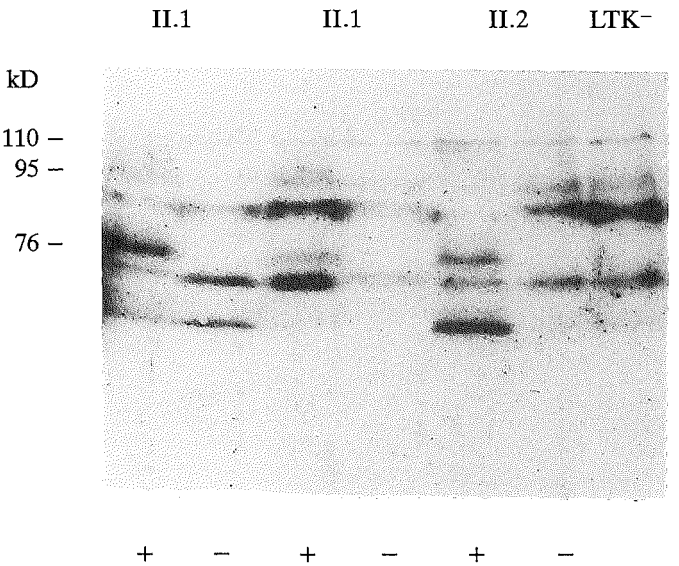

Figure 4. Immunoblot of acid α-glucosidase species in human-mouse somatic cell hybrids with (+) and without (-) human acid α-glucosidase activity (procedure as in Fig. 3). As shown in this figure, the antiserum used recognizes also mouse acid α-glucosidase. The apparent molecular mass of the known molecular species of human acid α-glucosidase is indicated.

illustrated in figure 2. The average background activity of the mouse LTK$^-$ cell line is set at 1 (Figure 2, LTK$^-$). The acid α-glucosidase activity in the human-mouse somatic cell hybrids is expressed as multiples of this value. The 13 hybrids originating from fusions of normal human fibroblasts with LTK$^-$ cells had on average an activity of 9.9 ± 5.2. The hybrid cell lines from I.1 formed two groups. Ten clones had similar activities as unfused LTK$^-$ cells and were thus completely deficient in human acid α-glucosidase activity. Nine clones, on the other hand, expressed an average enzyme activity of 4.7 ± 1.5. A similar distribution of positive and negative clones was 1observed with hybrids obtained from II.1 and II.2. Ten II.1 clones and five II.2 clones did not express human acid α-glucosidase activity, whereas 13 II.1 clones and 14 II.2 clones had acid α-glucosidase activities as measured in hybrids obtained after fusion of control fibroblasts with LTK$^-$ cells (on average, 12.5 ± 5.3 and 14.6 ± 6.6, respectively). Since the fibroblasts from cases of infantile glycogenosis type II in the third generation did not have measurable acid α-glucosidase activity, no effort was taken to use those for production of human-mouse somatic cell hybrids.

Analysis of acid α-glucosidase protein in human-mouse somatic cell hybrids

Immunoblotting was used as a second, independent, method to distinguish the products of different human acid α-glucosidase alleles. The molecular species observed in normal human fibroblasts (Figure 3, control) represent the precursor (110

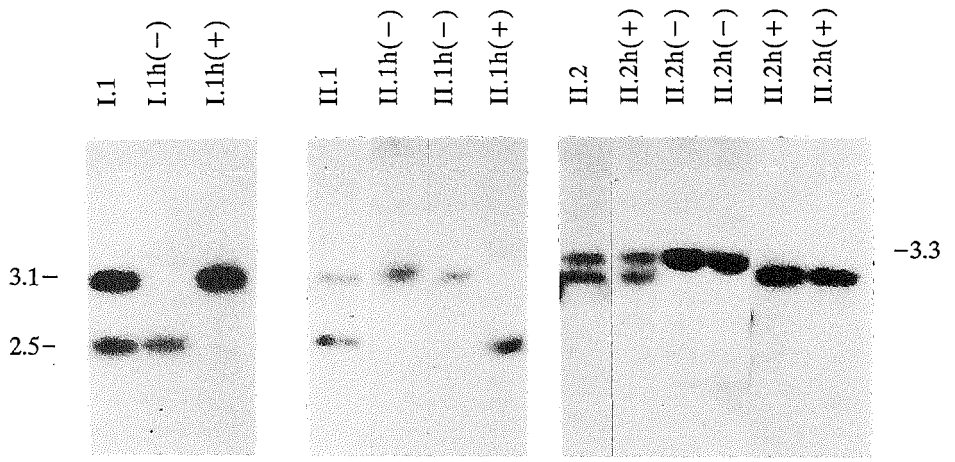

Figure 5. Identification of diferent human chromosomes 17 using the VNTR marker pYZN22. DNA was digested with TaqI and hybridized to probe pYNZ22 recognizing a variable number of tandem repeats localized on the p arm of human chromosome 17. The different chromosomes are in each case identified by a different length of the TaqI restriction fragments. Hybrids with (+) and without (-) human acid α-glucosidase expression are indicated.

kD), intermediate (95 kD) and mature (76 kD and 70 kD) forms of acid α-glucosidase (Hasilik and Neufeld, 1980; Reuser et al., 1985). The other lanes depict acid α-glucosidase of the family members. The immunoblot pattern of I.1, II.1 and II.2 can not be distinguished from normal immunoblot patterns. The amount of enzyme, however, is clearly reduced in I.1. A complete lack of mature enzyme is observed in fibroblasts of the three sibs III.1, III.2 and III.4 with severe infantile glycogenosis type II. Thus, the level of residual acid α-glucosidase activity in fibroblasts of the family members (Loonen et al., 1981a, 1981b) corresponds with the amount of mature enzyme that is formed. A similar correlation was observed in the hybrid cell lines (Figure 4). Mature human acid α-glucosidase of 76 kD was only detected in hybrid clones with human enzyme activity (+) and not in those without it (-). The expression of other molecular forms of human acid α-glucosidase in the hybrids was not informative due to cross-reactivity of the antiserum with mouse enzyme species with the same molecular mass.

DNA analysis of human-mouse somatic cell hybrids

A highly polymorphic chromosome 17 marker detectable with the VNTR probe pYNZ22 was used to demonstrate that human acid α-glucosidase-positive and −negative clones contained, indeed, different human chromosomes 17. In case I.1 and II.1 these were represented by two *Taq*I fragments of 3.1 and 2.5 kb, respectively

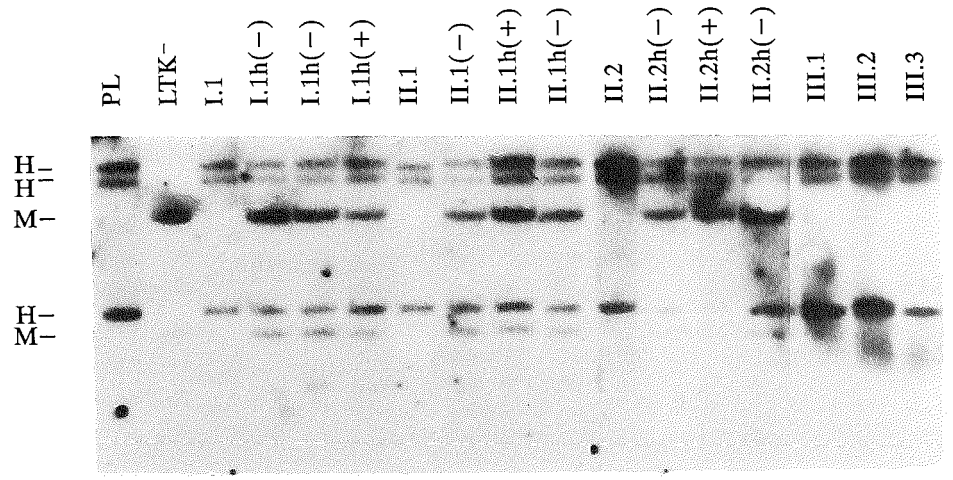

Figure 6. Hybridization analysis of genomic DNA isolated from human placenta (PL), mouse LTK$^-$ cells, fibroblasts of family members, and human-mouse somatic cell hybrids with (H(+)) and without (H(-)) human acid α-glucosidase activity. DNA was digested with HindIII and hybridized to human acid α-glucosidase cDNA. Hybridizing genomic DNA fragments from human and mouse origin are indicated with H and M, respectively.

(Figure 5). Hybrids derived from I.1 and II.1 contained only one of the two *Taq*I fragments and, therefore, had retained only one chromosome 17 which was different in human acid α-glucosidase-positive and -negative hybrids (see Figure 5 for a representative picture). The fact that the expression of human enzyme activity in hybrids of I.1 and II.1 is associated with different *Taq*I fragments illustrates that meiotic crossing-over has occurred. The different chromosomes 17 of II.2 were represented by *Taq*I fragments of 3.3 and 3.1 kb (Figure 5). The 3.3 kb fragment was found in all human acid α-glucosidase-negative hybrids (-) and the 3.1 kb fragment in all positive hybrids (+). One positive hybrid from the II.2 series contained both chromosomes 17, as indicated by the presence of two *Taq*I fragments (see Figure 5 for a representative picture). This was the only hybrid with two chromosomes 17 among the 21 hybrids tested.

Finally, the possibility had to be excluded that the lack of human acid α-glucosidase expression in the negative hybrids was due to loss of linkage between the TK and acid α-glucosidase gene loci. For this reason DNA of the different cell lines was digested with *Hin*dIII and hybridized with the radioactively labelled full-length cDNA coding for human acid α-glucosidase (Hoefsloot et al., 1988). Figure 6 shows that human DNA restriction fragments of identical size were detected in all hybrids, establishing the presence of a human acid α-glucosidase allele.

Conclusions

The differential expression of human acid α-glucosidase in the hybrids is determined by the nature of the mutant acid α-glucosidase allele that is retained. Patient I.1, who had mild clinical symptoms, is obviously a genetic compound. One allele does not lead to significant production of acid α-glucosidase (Figure 4, I.1 (-)) and is associated with complete enzyme deficiency. The other allele is characterized by reduced net production of acid α-glucosidase, as judged by the amount of enzyme present in fibroblasts of I.1 (Figure 3) and the level of activity in the positive clones of I.1 (40% compared to control). The reported activity of acid α-glucosidase in fibroblasts and various tissues of the grandfather (I.1) (see Materials and Methods) is in accordance with the expected combined effect of the two mutant alleles and can explain the mild clinical phenotype.

The allele with the most destructive mutation is transmitted to II.1, as indicated by the complete deficiency of acid α-glucosidase activity and protein in approximately 50% of the somatic cell hybrids derived from II.1. The normal activity in the other hybrid clones confirms that II.1 is a heterozygote. The estimated and actual acid α-glucosidase activity in cells, tissues and urine of II.1 is approximately half normal (Loonen et al., 1981a; 1981b) which is high enough to prevent glycogen accumulation and clinical symptoms (Reuser et al., 1987; Swallow et al., 1989). From the pedigree it must be concluded that II.2 also is a carrier of glycogenosis type II. Indeed, the

average acid α-glucosidase activity in cells and tissues of this family member is in the heterozygous range (Loonen et al., 1981a; 1981b). Analysis of somatic cell hybrids of II.2 showed that only one allele contributes to this activity (Figure 2). The mutant allele does not lead to enzyme production.

Obviously the three siblings in the third generation (III.1, III.2, III.4) have inherited the mutant alleles from the parents, which results in both an almost complete deficiency of acid α-glucosidase and a severe form of glycogenosis type II. Our present analysis does not reveal whether these cases are homo- or heteroallelic. Gross gene abnormalities were not detected (Figure 6, and authors' unpublished results), but different point mutations cannot be excluded. For instance, protein analysis indicates that several allelic mutations can cause the infantile subtype of this disease (Beratis et al., 1983; Reuser et al., 1985, 1987; Van der Ploeg et al., 1989a). In this respect the situation is not different from what is observed in other lysosomal storage diseases. Genetic heterogeneity has even been demonstrated in Gaucher disease type 1 (Tsuji et al., 1987; 1988) and in classical Tay-Sachs disease (Myerowitz and Hogikyan 1986, 1987; Arpaia et al., 1988), which were originally thought to be genetically homogeneous.

Presently, the awareness is growing that various combinations of homo- and heteroallelic mutant genotypes are the basis for clinical heterogeneity in lysosomal storage disorders. However, it is often difficult to correlate genotype and phenotype, because the attempt requires not only molecular but also functional analysis of the individual allele products. In this rather unique family with glycogenosis type II we had the opportunity to perform such an analysis. Compound heterozygosity was demonstrated in one of the cases, and strong support was obtained for the hypothesis that clinical heterogeneity in glycogenosis type II is primarily caused by allelic diversity at the acid α-glucosidase gene locus.

Materials and Methods

Family history

The pedigree of family S is illustrated in Figure 1. The index patient (III.1) was diagnosed at the age of 3 months with typical symptoms of infantile glycogenosis type II. She died at 16 weeks of age. Lysosomal α-glucosidase activity in skeletal muscle, liver and cultured fibroblasts was less than 1% of normal. Two more cases of severe infantile forms of the disease occurred in this generation. One child (III.2) was born immaturely after 25 weeks of gestation and lived for half an hour. Virtually no acid α-glucosidase activity was found in fibroblasts cultured from a skin biopsy taken post-mortem. The third incidence was an affected fetus (III.4) diagnosed prenatally with complete deficiency of acid α-glucosidase activity in cultured amniotic fluid cells. The diagnosis was confirmed after termination of pregnancy (Loonen et al., 1981a).

The parents are at present 43 (II.1) and 41 (II.2) years old, without clinical signs of glycogenosis type II. According to the autosomal recessive mode of inheritance of the disease, both parents must carry at least one mutant acid α-glucosidase allele. The levels of enzyme activity measured in skeletal muscle,

lymphocytes, cultured fibroblasts, and urine of both parents were about 50% of normal (Loonen et al., 1981a; 1981b).

The grandfather (I.1) did not experience symptoms of glycogenosis type II until the age of 53. When he was 59 years old the diagnosis of adult glycogenosis type II was established by the observation of lysosomal glycogen storage in a muscle biopsy and of reduced acid α-glucosidase activity in muscle tissue (16%), lymphocytes (17%), cultured fibroblasts (21%), and urine (13%). He died at the age of 72 from respiratory failure. On the basis of clinical and biochemical criteria, the spouse of I.1 is neither a patient nor carrier of the disease (Loonen et al., 1981a; 1981b).

Cell culture procedures and selection of hybrids

Cells were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories Inc. McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Human fibroblasts were fused with thymidine kinase (TK) deficient mouse L cells (LTK^-) using ß-propiolactone-inactivated Sendai virus as described (Westerveld et al., 1971). Human-mouse somatic cell hybrids were selected in HAT medium (Littlefield 1964). The aminopterine (A) present in this medium prevents the growth of thymidine kinase deficient mouse cells which are unable to utilize the added thymidine (T). Unfused human fibroblasts and human-human hybrids are outgrown by the rapidly proliferating human-mouse somatic cell hybrids. The human-mouse hybrid cell clones originating from each fusion event were isolated and propagated in HAT medium.

Assay of human acid α-glucosidase activity

Exactly the same procedure was used as described before (De Jonge et al., 1985). In short, human acid α-glucosidase was precipitated from cell homogenates with antibodies, raised in Swiss mice, against the enzyme purified from human placenta. The antibodies do not cross-react with mouse acid α-glucosidase. The activity of human acid α-glucosidase was measured in the immunoprecipitate with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside.

Immunoblotting

Different molecular species of acid α-glucosidase were separated electrophoretically in 10% polyacrylamide gels containing SDS (Laemmli, 1970), and blotted onto nitrocellulose filters (Towbin et al., 1979). The human and mouse enzymes were visualized using rabbit anti-human acid α-glucosidase serum in combination with ^{125}I protein A.

Southern blotting

To identify the acid α-glucosidase alleles, DNA was isolated from fibroblasts and hybrid cell lines by using standard methods (Maniatis et al., 1982). Following digestion with the restriction enzyme *Hin*d III, DNA fragments were separated in 0.7% agarose gels. After blotting onto nitrocellulose (Maniatis et al., 1982), filters were hybridized with a full-length acid α-glucosidase cDNA probe (Hoefsloot et al., 1988) radioactively labeled according to the method described by Feinberg and Vogelstein (1983). Filters were washed after 16 hours till a stringency of 0.3 times SSC (1 x SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and were exposed to Fuji X-ray film for 1-4 days.

The same technical procedures were used for the identification of the different chromosomes 17 in fibroblasts from the family members and in the clonal human-mouse cell hybrids. For this purpose the DNA was cut with *Taq*I and hybridized with probe pYNZ22 recognizing a DNA segment on human chromosome 17 with a variable number of tandem repeats (HGM locus D17S30; Nakamura et al., 1987, 1988).

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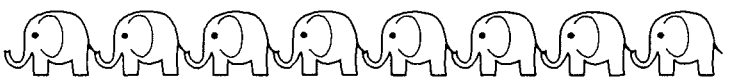
CHAPTER 8



Glycogenosis type II: Protein and DNA analysis in five South African families from various ethnic origins[1]

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Summary

The molecular nature of lysosomal α-glucosidase deficiency was studied in five South African families with glycogenosis type II. Distinct ethnic origins were represented. Two new mutant acid α-glucosidase alleles were discovered. In two infantile patients from a consanguineous Indian family we found for the first time an acid α-glucosidase precursor of reduced size. The mutant precursor appeared normally glycosylated and phosphorylated, but was not processed to mature enzyme. Abnormalities of the mRNA were not obvious, but digestion of genomic DNA with *Hin*dIII, *Bgl*II and *Stu*I revealed for each enzyme a fragment of increased length. Heterozygosity was demonstrated in the parents. Complete lack of acid α-glucosidase mRNA, as well as deficiency of precursor synthesis, was observed in two black baby girls from unrelated families. In these cases the length of all restriction-enzyme fragments was normal. Reduced enzyme synthesis but normal processing was registered in juvenile and young adult Cape Colored patients. The extensive heterogeneity of glycogenosis type II is emphasized in these studies on various ethnic groups. The newly discovered mutants are valuable for the understanding of clinical diversity as a result of allelic variation.

Introduction

Acid α-glucosidase is a lysosomal hydrolase essential for the degradation of glycogen (Hers 1963). Enzyme deficiency leads to glycogenosis type II and is inherited as an autosomal recessive trait (Howell and Williams 1983). Three clinical variants are distinguished (Hers and De Barsy 1973). The infantile subtype has its onset shortly after birth and presents with general hypotonia and muscle wasting. Cardiorespiratory insufficiency leads to death in the first 2 years of life. The adult variant manifests itself mostly after the second decade of life and is characterized by

[1]Adapted from Am. J. Hum. Genet. 44:787-793 (1989)

progressive skeletal muscle weakness. Respiratory distress is ultimately fatal. Often, a juvenile subtype is distinguished as an intermediate variant. A fairly strict correlation exists between clinical severity of the disease, extent of lysosomal glycogen storage, and residual activity of acid α-glucosidase in cultured fibroblasts and muscle cells of patients (Reuser et al., 1978, 1987; Van der Ploeg et al., 1987, 1988a, 1988b). The biosynthesis of lysosomal α-glucosidase is a complex process, whereby a precursor with an apparent molecular mass of 110 kD is gradually converted to mature species of 76 kD and 70 kD (Hasilik and Neufeld 1980a, Oude Elferink et al., 1985; Reuser et al., 1985). This posttranslational processing involves modification of oligosaccharide side-chains and proteolytic trimming of the precursor at both the amino- and carboxyterminal end (Van der Horst et al., 1987; Hoefsloot et al., 1988). Among the patients with glycogenosis type II, mutants have been found with reduced synthesis of acid α-glucosidase or with catalytically inactive enzyme. Furthermore, there were variants with defective phosphorylation of the precursor and/or impaired processing and intracellular transport. The latter studies concerned mainly patients of Dutch ancestry (Reuser et al., 1985, 1987). We presently report on genetic heterogeneity of glycogenosis type II in the South African population. Three families and two individual cases were studied. Interesting new acid α-glucosidase mutations were discovered.

Case Reports

Family A

This Cape colored family (Figure 1A) of Dutch ancestry was referred because of severe skeletal muscle weakness in the first proband of the second marriage of the mother. He was a thin and weak 25-year-old man who had not been able to work for the past 5 years, owing to muscle wasting. At home he needed both assistance to get out of bed and support in walking. Chest X-ray and electrocardiogram (ECG) were normal. Pompe disease was suspected and diagnosed on skeletal muscle biopsy and fibroblast α-glucosidase assay. Urine samples of the eight siblings were tested by thin layer chromatography, and revealed that two younger brothers of 11 and 13 years old were also affected (Sewell 1979, Blom et al., 1983). Till then no gross abnormalities had been noted by the parents. On clinical examination a positive Gower sign was found in the elder boy. The only possible indication for Pompe disease in the younger proband was his statement that he could not run as fast as other boys in his class. Skin biopsies were obtained both from the two younger boys and from the parents, for growth of fibroblasts.

Family B

This was a consanguineous Cape colored family (Figure 2A). The oldest son was

admitted at the age of 4 years with severe muscle weakness and an abnormal gait. A myopathic electromyogram and raised creatine phosphokinase were found, whereas echocardiogram and electrocardiogram were essentially normal. Glycogen storage was demonstrated in skeletal muscle tissue, and acid α-glucosidase deficiency was measured in cultured fibroblasts. At the time that Pompe disease was diagnosed, the mother was in her third pregnancy, too late for prenatal diagnosis. Since the family lives in a remote country area, follow-up was not possible.

Family C

The 4 months old female proband of this consanguineous Indian family (Figure 3A) was referred because of poor feeding and failure to thrive (Bonnici et al., 1980). Motor development was delayed. She lay virtually immobile and was crying incessantly in a weak voice. A severe generalized hypotonia, severe tachypnea and a prominent left hemithorax were noticed. Macroglossia was not present, and liver enlargement was moderate. Tendon reflexes were absent. The chest X-ray revealed massive cardiac enlargement. The EKG showed a short P-R interval and gigantic QRS complexes in leads S_1 and S_2 and the left chest leads. The axis was +45°. Pompe's disease was diagnosed on cultured fibroblasts of the patient. The family decided against prenatal diagnosis, and a second affected infant was born. A following pregnancy was monitored, and an unaffected child was born.

Case D

This was a black Zulu baby girl from the Durban area on the east coast of South Africa. She was referred at the age of 7 months with severe hypotonia, cardiomegaly, and liver enlargement. The muscle biopsy showed a morphology characteristic of Pompe's disease. No family history is available.

Case E

This black Ovambo baby girl from South West Africa (Namibia) was, at admission, moribund with gross respiratory distress syndrome, cardiomegaly, and hypotonia. Skeletal muscle biopsy was suggestive for glycogen storage, and acid α-glucosidase deficiency was measured in cultured fibroblasts and Epstein-Barr virus-transformed lymphoblasts. No family history is available.

Results

Acid α-glucosidase activities were measured in cultured fibroblasts of the various patients and in some of the parents under standardized conditions (Table 1). The enzyme deficiency was nearly complete in the severe infantile cases (C.III.3 and C.III.4, and cases D and E), whereas in the young adult and juvenile cases (A.II.6,

A.II.7 and B.III.1) a residual acid α-glucosidase activity of 9%-13% of the lowest control value was measured. In fibroblasts derived from the parents, enzyme activities were within or slightly below the control range.

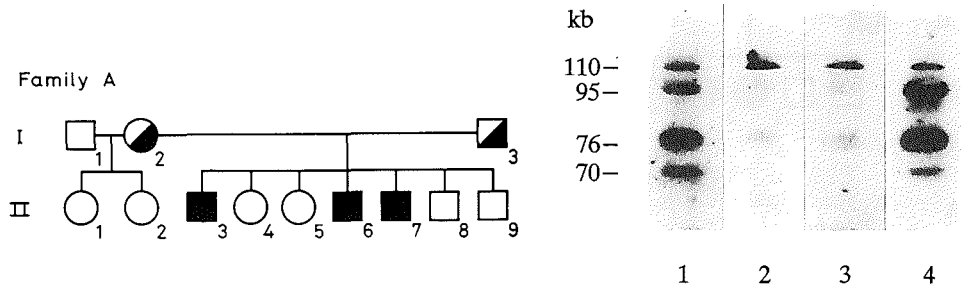
Table 1. Acid α-glucosidase activities

Subject(s)		MU[a]	Glucose[b]/MU[a]
Family A	I.2	58.6	6.8
	I.3	95.6	9.2
	II.6	9.1	8.0
	II.7	7.5	7.7
Family B	III.1	6.9	5.0
Family C	I.3	77.9	9.0
	II.1	78.5	8.0
	III.3	0.5	11.0
	III.4	0.65	6.3
Case D		0.67	9.1
Case E		0.51	10.4
Control (n=10)		70-150	

[a]nmol 4-methylumbelliferone/mg protein/h
[b]nmol glucose/mg protein/h

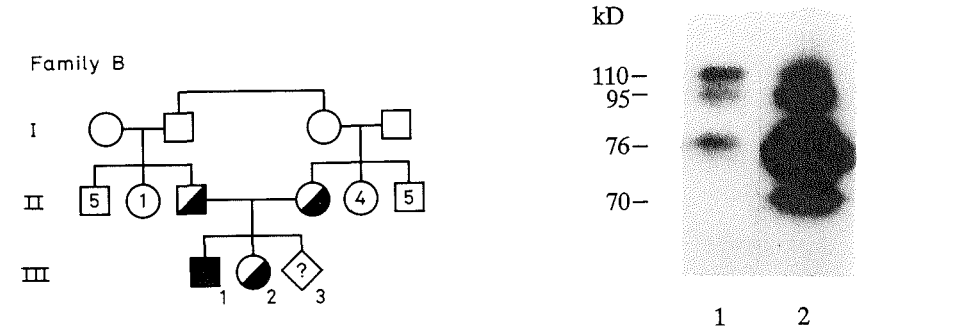
In order to analyse the nature of the defect in the various cases, the molecular forms of lysosomal α-glucosidase were separated by SDS-PAGE and were visualized immunologically after transfer to nitrocellulose. Figure 1B shows the pattern obtained for family A. In both parents (Figure 1B, lanes 1 and 4) and the affected children (Figure 1B, lanes 2 and 3) acid α-glucosidase is processed normally from a 110 kD precusor via a 95 kD intermediate to mature 76 kD and 70 kD enzyme. However, the amount of protein in both patients appears to be very much reduced. An identical picture is obtained for mutant B.III.1 (Figure 2B).

In family C, another defect in acid α-glucosidase synthesis is observed. Instead of a 110 kD precursor, a protein of slightly lower molecular weight (approximately 105 kD) shows on the immunoblot of the probands (Figure 3B, lanes 4 and 5). This aberrant molecular weight species is also present in cells from the consanguineous parents, but normally processed enzyme is seen in addition (Figure 3B, lanes 3 and 6). The 110 kD precursor was, on this particular immunoblot, only detectable in cells of a patient with infantile glycogenosis type II, applied in lane 1 as marker. The biosynthesis of the mutant acid α-glucosidase was further investigated by in vitro



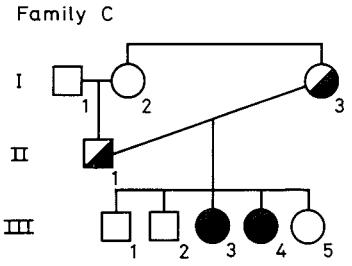
A B

Figure 1. A. Pedigree of family A. Filled symbols denote glycogenosis type II patients; half filled symbols denote obligate carriers; open symbols denote healthy individuals. B. Immunoblot of the biosynthetic forms of acid α-glucosidase in fibroblasts from members of family A. Lane 1, A.I.2 (mother); lane 2, A.II.6 (patient); lane 3, A.II.7 (patient); lane 4, A.I.3 (father).

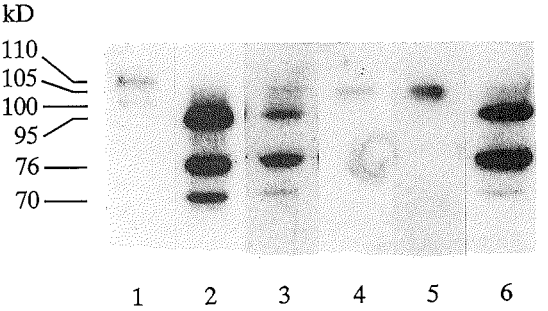


A B

Figure 2. A. Pedigree of family B. The open diamond with a question mark denotes that the outcome of pregnancy is unknown. Figures in symbols indicate number of siblings. B. Immunoblot of the biosynthetic forms of acid α-glucosidase in fibroblasts. Lane 1, patient B.III.1; lane 2, healthy individual.


Family C
I
II
III
1
2
3
4
5

A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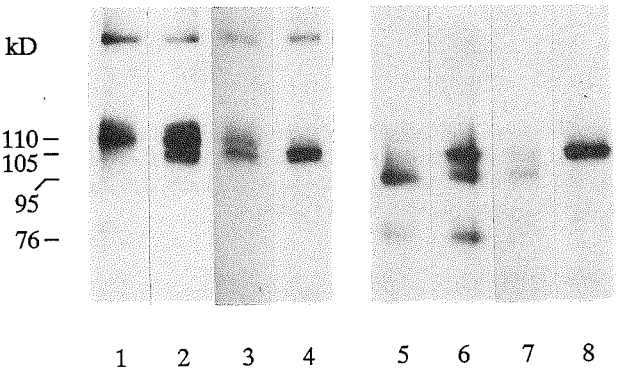
B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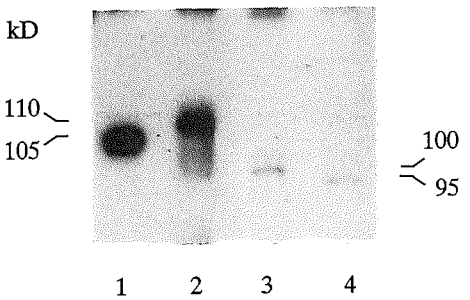
C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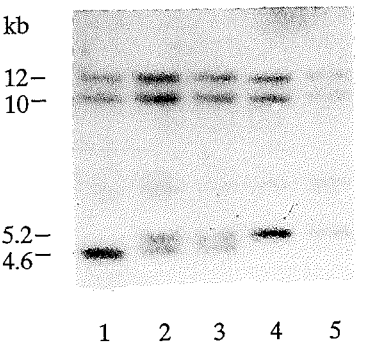
D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E

labeling with ^{3}H-leucine. The results obtained from 3-h pulse (Figure 3C, lanes 1-4) confirm that the 105 kD protein is indeed the first precursor synthesized in cells from the patients (Figure 3C, lane 4). A subsequent chase period of three hours revealed that the mutant protein is not further processed (Figure 3C, lane 8). This is also observed in cells from the parents (Figure 3C, lanes 2,3,6 and 7). The aberrant acid α-glucosidase band remains visible, whereas the normal precursor is processed as in control cells.

When labeling is performed in the presence of tunicamycin, to inhibit glycosylation, both the normal and the mutant precursor are reduced in size, but the difference in apparent M_r is maintained (Figure 3D). The aberrant 105 kD acid α-glucosidase precursor occurred to be normally phosphorylated (results not shown).

Since the abnormal size of the mutant precursor does not seem to reflect a processing defect, it may result from a gene deletion. To test this hypothesis, DNA from the patients and from their parents was digested with several restriction enzymes and hybridized with full-length cDNA of human acid α-glucosidase. Digestion of genomic DNA with the restriction enzyme *Hin*dIII reveals, in control cells, fragments of 12, 10 and 4.6 kb (Figure 3E, lane 1). In the patient cell lines the 12 and 10 kb fragments were normally present, but the 4.6 kb fragment was enlarged to 5.2 kb (Figure 3E, lanes 4 and 5). In cells from the parents the 4.6 as well as the 5.2 kb fragments were detected (Figure 3E, lanes 2 and 3). Larger DNA fragments were also observed when the genomic DNA was digested with the restriction enzymes *Bgl*II and *Stu*I, but not with *Eco*RI (not shown). Size abnormalities of α-glucosidase mRNA were not obvious on Northern blots from patients and parents (not shown). The amount of mRNA appeared also normal.

In neither of the unrelated black baby girls (Case D and E) was acid α-glucosidase protein detectable on immunoblots. Nor could biosynthetic forms of acid α-glucosidase be visualized by means of radioactive labeling of cells from the two infants (Figure 4, lanes 4 and 5). Figure 4 shows, for comparison, labeling of the 110 kD precursor from the young adult patient A.II.6 (Figure 4, lane 2), and the shorter precursor of infantile patient C.III.3 (Figure 4, lane 3). A Northern blot analysis of

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Figure 3. A. Pedigree of family C. B. Immunoblot of the biosynthetic forms of acid α- glucosidase in fibroblasts. Lane 1, infantile Italian glycogenosis II patient; lane 2, healthy individual; lane 3, C.I.3 (mother); lane 4, C.III.3 (patient 1); lane 5, C.III.4 (patient 2); lane 6, C.II.1 (father). C. Labeling, with ^{3}H leucine, of acid α-glucosidase in fibroblasts of family C. Cells were labeled for 3 h (lanes 1-4) and were chased for another 3 h (lanes 5-8). Lanes 1 and 5, control; lanes 2 and 6, mother; lanes 3 and 7, father; lanes 4 and 8, patient 1. The band near the kD label is not related to acid α-glucosidase. D. Labeling with ^{3}H-leucine, of acid α-glucosidase in the presence (+) or absence (-) of tunicamycin. Cells were labeled for 3 h. Lane 1, patient (-); lane 2, control (-); lane 3, patient (+); lane 4, control (+). E. Southern blot of HindIII digested fibroblast DNA, of family C, hybridized with radioactively labeled full-length cDNA of acid α-glucosidase. Lane 1, control; lane 2, mother; lane 3, father; lane 4, patient 1; lane 5, patient 2.

RNA extracted from fibroblasts of cases D and E indicates complete deficiency of acid α-glucosidase mRNA, while no gross gene deletion is observed on Southern blots.

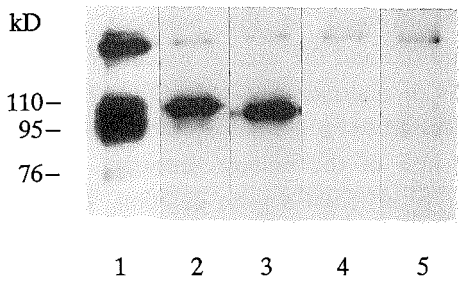

Figure 4. Pulse labeling of acid α-glucosidase with ^{3}H leucine for 6 h. Lane 1, control; lane 2, patient A.II.6; lane 3, C.III.3; lane 4, case D; lane 5, case E.

Discussion

The present study on the molecular background of glycogenosis type II in South African families supports our hypothesis that the clinical phenotypes of this disease correlate with the residual activity of lysosomal α-glucosidase (Reuser et al., 1987, Van der Ploeg et al., 1987, 1988). As in most juvenile and adult cases investigated in the past, synthesis and maturation of the mutant enzyme is observed in young adult and juvenile South African patients (A.II.6, A.II.7 and B.III.1). The amount of mature enzyme is, however, clearly reduced, which accounts for the decreased acid α-glucosidase activity in the patient's cells. The specific activity of the mutant enzyme is estimated to be close to normal. Also, the ratio of acid α-glucosidase activity for glycogen to activity for the artificial substrate 4- methylumbelliferyl-α-D-glycopyranoside is not significantly abnormal (Reuser et al., 1982, 1985, 1987). The fact that patient B.III.1 was born of consanguineous parents makes it likely that he carries two identical mutant alleles. This true homozygosity will facilitate molecular analysis of one of the mutations that predisposes for juvenile glycogenosis type II.

In a second consanguineous marriage of parents from Indian extraction, we identified for the first time a mutant α-glucosidase precursor of reduced size. Since the mutant enzyme is also shorter when synthesized in the presence of tunicamycin, it is likely that the abnormallity wich inhibits further processing resides in the protein core. The type of mutation in this particular family is not easily explained. In seeming

contradiction to the nature of the protein defect, a longer DNA fragment of 5.2 kb instead of 4.6 kb is observed when DNA of the patients is digested with *Hin*dIII. DNA fragments of abnormal length are also detected with the enzymes *Bgl*II and *Stu*I. The single *Eco*RI fragment seems unaltered, but this may be due to its large size of 20 kb. Among 20 Caucasians we did not encounter in the acid α-glucosidase locus a polymorphism that leads to the unusual fragment size (unpublished results). Therefore, the abnormalities seen at the protein and DNA levels may very well be related, but more detailed analysis will be needed befor firm conclusions can be drawn.

The second rather unique mutation that was observed in the present study is the complete lack of precursor synthesis in fibroblasts from the two black baby girls. Also, the amount of acid α-glucosidase mRNA was below detection. Lack of mRNA has once been described by Martiniuk et al. (1986), but the ethnic origin of this latter patient was not mentioned. At the DNA level no gross deletions were observed using various restriction enzymes. This suggests the occurrence of either a point mutation or a very small deletion, which blocks transcription or gives rise to an unstable mRNA.

In a random selection of 5 families from South Africa (various ethnic origins) in which glycogenosis type II occurred, we found extensive heterogeneity and interesting mutations. These can provide important information about the molecular prerequisites for synthesis and post-translational modification of lysosomal acid α-glucosidase. It seems worthwhile to extend these studies to distinct ethnic groups.

Materials and methods

Cell culture

Fibroblasts were cultured in Dulbecco's modified Eagle's Medium (DMEM, Flow Laboratories) supplemented with 10% FCS (Boehringer Mannheim) and antibiotics, in an atmosphere of 10% CO_2 and 90% air. One-week confluent cultures were harvested with trypsin and homogenized by sonication in 250 μl distilled water. Cell debris was removed by centrifugation at 10,000 g for 15 min.

Biochemical assays

Acid α-glucosidase activity was measured with the artificial substrate 4-methylumbelliferyl-α-D-glycopyrano-side (Galjaard 1980) or with glycogen (Koster et al., 1972). Protein concentrations were determined according to the method of Lowry et al., (1951).

Immunoblotting

Cell lysates (4 x 10^6 cells/200 μl, equivalent to 0.6-1 mg protein/200 μl) were incubated overnight at 4°C with 100 μl of a 1:1 suspension of concanavalin A sepharose 4B beads in sodium phosphate buffer, pH 6.2, with a final concentration of 20 mM. Nonspecifically bound proteins were removed by washing the beads four times with 1 ml of the same buffer. Bound glycoproteins were dissolved by heating for 10 min at 90°C in 75 μl of sample buffer (125 mM Tris-HCl, pH 6.6, 2 M glycerol, 4% SDS, 0.6% mercaptoethanol, and 0.05% bromophenol blue). Samples were electrophoresed in a 10% polyacrylamide gel (Laemmli 1970) and subsequently were blotted onto nitrocellulose (Towbin et al., 1979). Acid

α-glucosidase proteins were visualized with a rabbit polyclonal antibody preparation against human placental acid α-glucosidase (Reuser et al., 1985) in combination with ^{125}I-labeled protein A (Towbin et al., 1979).

In vivo labeling

Fibroblasts were cultured in leucine- or phosphate-free medium DMEM with addition of L-(4,5-^{3}H) leucine or carrier-free ^{32}P-phosphate, respectively (Reuser et al., 1985). In some experiments tunicamycin (final concentration 10 μ/ml) was added 3 h before addition of radioactive precursors to inhibit glycosylation of newly synthesized acid α-glucosidase. Cell homogenates were prepared, and acid α-glucosidase was immunoprecipitated with antibodies. Samples were subsequently analyzed in a 10% polyacrylamide gel according to method described elsewhere (Hasilik and Neufeld 1980a, 1980b; Reuser et al., 1985).

Analysis of RNA and DNA

DNA was extracted from cultured fibroblasts by following standard procedures and was digested with various restriction enzymes. DNA fragments were separated in 0.5-1% agarose gels and were transferred to Genescreen Plus™ (Southern 1975; Rigaud et al., 1987). The lithium chloride technique was used to isolate mRNA (Auffray and Rougeon, 1980). The RNA was electrophoresed in 1% agarose/formaldehyde gels and was blotted onto nitrocellulose. Filters were hybridized with radioactively labeled acid α- glucosidase cDNA probes (Maniatis et al., 1982; Feinberg and Vogelstein 1983; Hoefsloot et al., 1988). After 16 h the filters were washed to a stringency of 0.3 x SSC and were autoradiographed.

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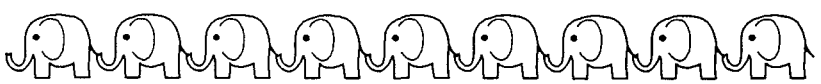
CHAPTER 9



Lysosomal α-glucosidase: RFLP's and mutant alleles in the asian population[1]

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Summary

Deficiency of lysosomal (acid) α-glucosidase is the primary cause of glycogenosis type II. Clinical heterogeneity has been attributed to the expression of different mutant alleles at the acid α-glucosidase gene locus (GAA). We have previously studied five unrelated cases of glycogenosis type II in the mixed South African population. With acid α-glucosidase cDNA as a probe we discovered two patients with DNA restriction fragments of unusual length. These patients were of Indian descent. We presently report on a second such situation in a Taiwanese family with glycogenosis type II, but a causal relationship between the unusual restriction fragment pattern and the lysosomal α-glucosidase deficiency is excluded. Instead, an acid α-glucosidase polymorphism is demonstrated to occur in the Asian population which so far has not been encountered among Caucasians. This polymorphism is detectable with several restriction enzymes like *Hin*dIII, *Bgl*II, and *Stu*I, and is caused by an insertion c.q. deletion of approximately 600 bp in the region covering exon 10 to 16 of the lysosomal α-glucosidase gene. Other alleles at the acid α-glucosidase locus are identified as *Eco*RI and *Xba*I RFLP's with similar allele frequencies in the Asian and the Caucasion population. Mutant acid α-glucosidase alleles of patients from various ethnic origins are distinguished by Northern blot analysis, or are identified as abnormalities in enzyme synthesis. These studies exemplify the genetic heterogeneity in glycogenosis type II.

Introduction

Lysosomal α-glucosidase (E.C. 3.2.1.3) is encoded by the GAA locus on

[1]submitted

chromosome 17q22-q23 (Solomon et al., 1979; Sandison et al., 1982; de Jonge et al., 1984; Martiniuk et al., 1985). Four alleles at this locus have been identified by analyzing the physical properties of the enzyme in the Caucasian and Malayan Indian population (Nickel and McAlpine, 1982; Swallow et al., 1975, 1989). Twenty others were discovered more recently by cDNA and gene sequence analysis, or as RFLP's (Hoefsloot et al., 1988, 1990a, 1990b, 1991; Martiniuk et al., 1990a, 1990b; Tzall et al., 1990a, 1990b). In addititon to these "wild type" alleles, there are at least the same number of mutant alleles. These were partly identified by Northern blot analysis, and in part by studying the synthesis and post-translational modification of lysosomal α-glucosidase in cells and tissues from patients with an enzyme deficiency associated with glycogenosis type II (Reuser et al., 1978, 1985, 1987, 1988; Reuser and Kroos, 1982; Beratis et al., 1983; Hoefsloot et al., 1988, 1990d; Van der Ploeg et al., 1988, 1989a). The clinical heterogeneity in this storage disease has been attributed to homozygozity or compound heterozygozity for different mutant acid α-glucosidase alleles (Reuser et al., 1987, 1988; Hoefsloot et al., 1990d).

Glycogenosis type II has been studied most extensively in the Caucasian population in which it occurs with an incidence of approximately 1 in 150.000 (McKusick, 1988). But the disease has a wider ethnic and geographic distribution. Arabic, Black, Chinese, Indian, and Japanese cases have been reported (Beratis et al., 1983; Trend et al., 1985; Lin et al., 1987; Ninomiya et al., 1984; Usuki et al., 1988; Tanaka et al., 1979; Iancu et al 1988; Van der Ploeg et al., 1989a). In a recent study we described the occurrence of glycogenosis type II in the mixed South African population, and we reported on a novel mutant phenotype in the offspring of consanguineous parents of Indian descent (Van der Ploeg et al., 1989). The two patients in this family were shown to produce a smaller then normal lysosomal α-glucosidase precursor. Seemingly in contrast to this finding, Southern blot analysis revealed larger than normal DNA fragments upon digestion with *Hin*dIII, *Bgl*II and *Stu*I. The parents were heterozygous having both the normal and the larger size DNA fragments. It was questioned as to whether the abnormal protein and DNA patterns were related or whether the unusual restriction fragment pattern represented a hitherto undiscovered polymorphism. The question is addressed in this paper which also reports on the properties of mutant lysosomal α-glucosidase in Taiwanese families with glycogenosis type II.

Results

The uncommon *Hin*dIII restriction fragment pattern in a South African family with glycogenosis type II is illustrated in Figure 1. The healthy individuals in this panel (homozygous for either the GAA1 or GAA2 allele) and several patients with infantile or adult forms of glycogenosis type II have the common *Hin*dIII pattern with

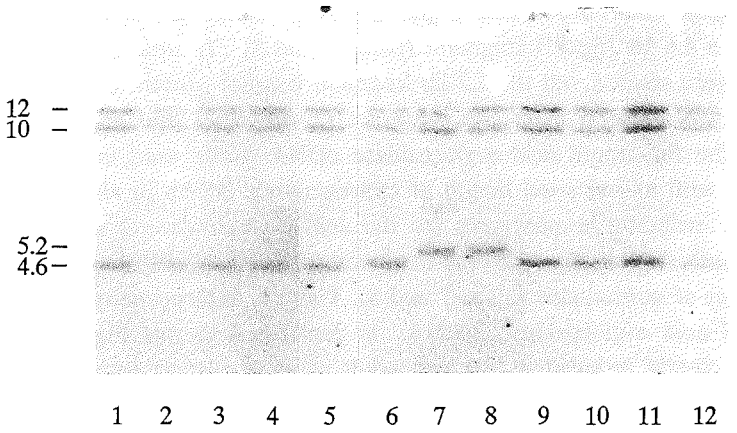

Figure 1. Southern blot analysis of HindIII digested DNA from healthy individuals (C) and patients with infantile (I) or adult (A) glycogenosis type II. Full-length acid α-glucosidase cDNA was used as a probe. Lane 1, 86RD698 (C); lane 2, 82RD167 (A); lane 3, AS (A); lane 4, 217LAD (I); lane 5, 266LAD (I); lane 6, 85-48 (I); lane 7, 78-14 (I); lane 8, 81-1 (I); lane 9, 82-4 (I); lane 10, HM (C, homozygous for GAA2 allele); lane 11, Cyst L (homozygous for GAA2 allele); lane 12, Cyst R (homozygous for GAA1 allele). The estimated length of the DNA fragments is given in kilo bases (kb).

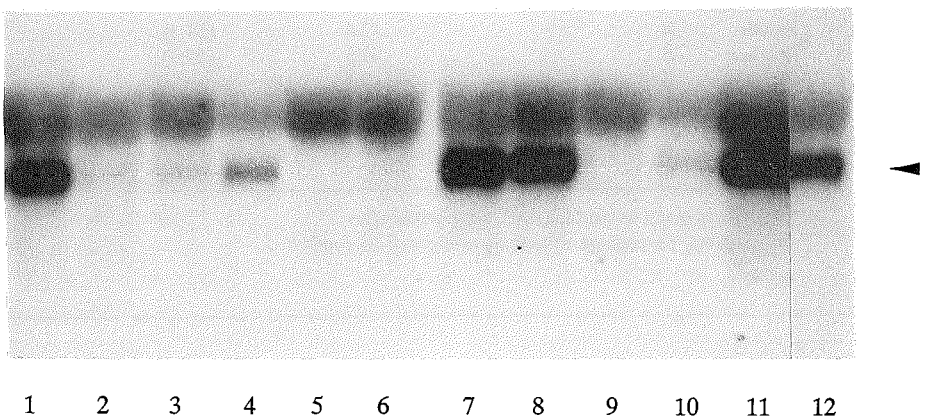

Figure 2. Northern blot analysis of acid α-glucosidase mRNA in fibroblasts from healthy individuals (C) and patients with infantile (I) or adult (A) glycogenosis type II. Full-length acid α-glucosidase cDNA was used as a probe. The order of the lanes is the same as in Fig 1. The arrow head points to the acid α-glucosidase messenger with an estimated size of 3.8 kb. The second hybrizing band (28 S ribosomal RNA) can be used to compare the amount of RNA in each lane.

fragments of 12, 10 and 4.6 kb. The two previously described South African patients with a smaller than normal acid α-glucosidase precursor are shown to have a 5.2 kb instead of a 4.6 kb *Hin*dIII fragment (lanes 7 and 8) . In total 26 healthy or affected Causians were studied, but the 5.2 kb fragment was not present in any of these cases.

RNA was isolated from the same set of cell lines and analyzed by Northern blotting. The full-length acid α-glucosidase cDNA probe used in Figure 2 detects a messenger with an expected length of approximately 3.8 kb in all samples of healthy individuals including homozygotes for the acid α-glucosidase 2 allele (GAA2). Also the two South African sibs with the uncommon 5.2 kb *Hin*dIII fragment seem to have a messenger of normal size (lanes 7 and 8). There is heterogeneity with respect to the amount of acid α-glucosidase mRNA. As illustrated in this Figure the amount of mRNA is clearly reduced in the two adult cases of glycogenosis type II (Figure 2, lanes 2 and 3). Of the infantile patients, the two South African sibs with the 5.2 *Hin*dIII allele express a normal amount of mRNA (lanes 7 and 8). In four other infantile cases, however, the amount of mRNA seems to be reduced or acid α-glucosidase mRNA is undetectable (lanes 4, 5, 6 and 9).

Table 1. Acid α-glucosidase activity in cultured fibroblasts

Cell line		Activity*	
Control 1		84.5	
Control 2		79.7	
Control 3		82.8	
Father	B	42.4	(51.3)
Mother	B	49.5	(59.9)
Patient	B	0.1	(0.1)
Father	R	45.8	(55.4)
Mother	R	19.1	(23.1)
Patient	R	0.07	(0.08)

*Activity is given as nmoles 4MU/mg protein/h
In brackets, percentage of activity compared to control

The significance of the 5.2 kb *Hin*dIII fragment for the occurrence of glycogenosis type II was elucidated when in a separate series of experiments the molecular cause of acid α-glucosidase deficiency was studied in two Taiwanese families (R and B). A case of infantile glycogenosis type II had been diagnosed in both these families. An extremely low residual enzyme activity of less than 0.1 % was measured in fibroblasts of the affected family members (Table 1). Immunoblot analysis was performed to

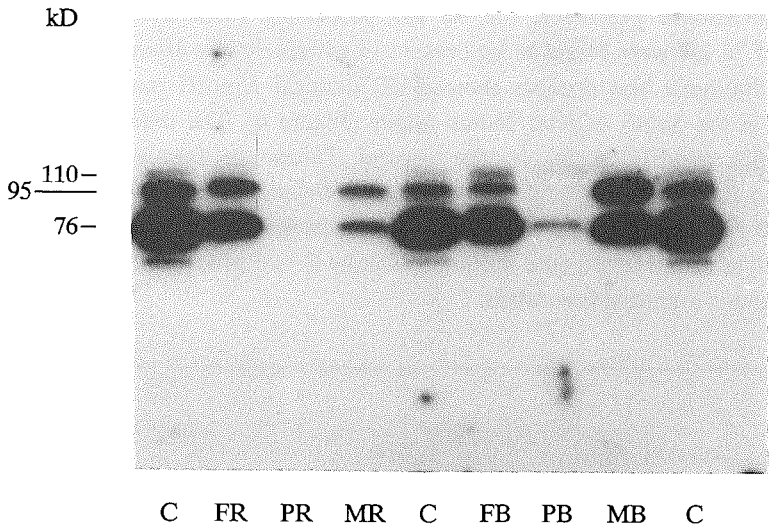

Figure 3. Immunoblot analysis of the steady state level of the precursor and the mature forms of acid α-glucosidase in two Taiwanese families with glycogenosis type II. Lysosomal α-glucosidase was immunoprecipitated from cell homogenates and precursor and mature enzyme species were separated on SDS-PAGE. Proteins were transferred to nitrocellulose filters and visualized with polyclonal rabbit antiserum in combination with ^{125}I-protein A and fluorography. Panel R, family R; panel B, family B. Family members are indicated with the letters F (father), M (mother) and P (patient); the letter C stands for a control cell line (86RD698).

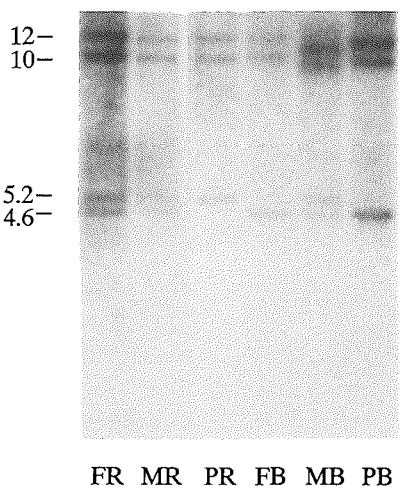

Figure 4. Southern blot analysis of HindIII digested DNA from members of two Taiwanese families with glycogenosis type II. Panel R, family R; panel B, family B. Family members are indicated with the letters F (father), M (mother) and P (patient).

further characterize the defect (Figure 3). In fibroblasts of both patients the 110 kD acid α-glucosidase precursor, the 95 kD processing intermediate and the mature enzyme of 76 kD were found to be present in extremely low amount.

The Southern blot analysis showed an identical *Hin*dIII pattern in family R as observed in the South African/Indian family (Figure 4). The patient was homozygous for the 5.2 *Hin*dIII allele. In the second Taiwanese family (B), however, the Mendelian inheritance of acid α-glucosidase deficiency appeared not to be linked to the 5.2 kb *Hin*dIII allele but to the more common 4.6 kb allele (Figure 4). Therefore, the 5.2 allele of the unaffected mother in family B had to represent a wild type rather than a mutant α-glucosidase allele.

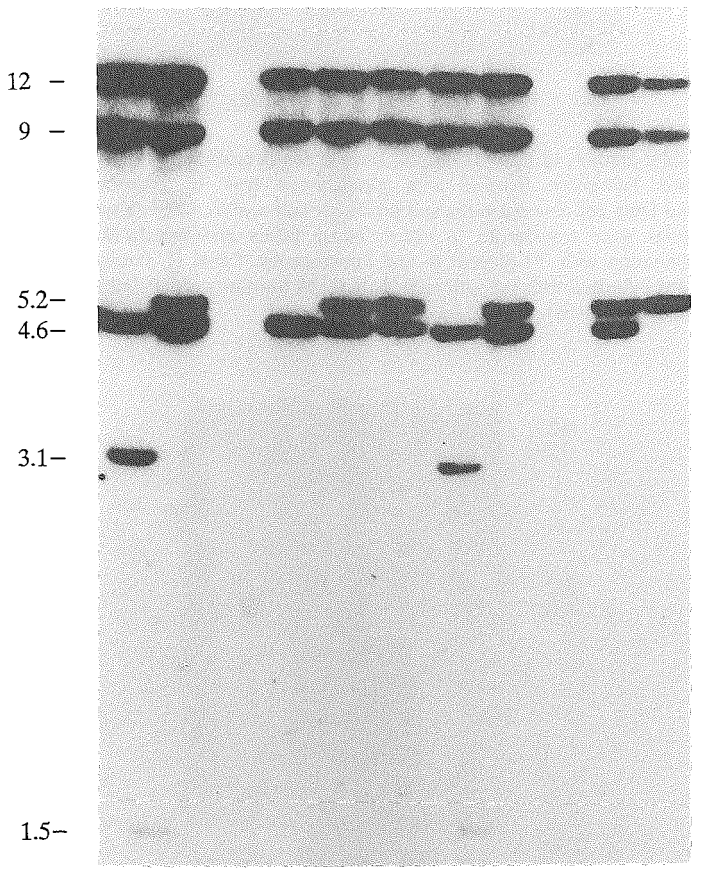

Figure 5. Southern blot analysis of HindIII/EcoRI double digested DNA from a pannel of healthy individuals from Japan. Full-length acid α-glucosidase cDNA was used as a probe. The estimated length of the DNA fragments is given in kilo bases (kb).

These results prompted us to investigate the possible occurrence of a *Hin*dIII polymorphism in the Asian population as opposed to the Caucasion population. In a further series of experiments the DNA was analyzed of 12 Dutch inhabitants originating from Suriname (from Chinese and/or Indonesian descent) and from mainland China. Among this panel the 4.6 kb *Hin*dIII fragment was encountered 6 times in homozygous form, while 5 individuals were found to be genetic compounds with both the 4.6 kb and the 5.2 kb *Hin*dIII alleles. Homozygozity for the *Hin*dIII 5.2 kb allele was encountered only once (result not shown).

To extend this survey to other Asian populations and to obtain a more accurate estimate of the incidence of the *Hin*dIII polymorphism we analyzed the DNA of 18 healthy Japanese individuals. This panel was used to estimate at the same time the incidence of an *Eco*RI polymorphism previously discovered in the Caucasian population. The polymorphic *Eco*RI site is not readily detectable by digestion of DNA with *Eco*RI alone since the polymorphism is identified by only a subtle 1.5 kb

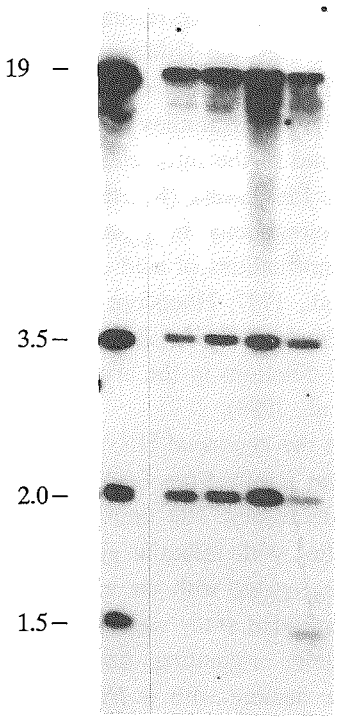

Figure 6. Southern blot analysis of XbaI digested DNA from a panel of healthy individuals from Japan. (see Figure 5 for further details).

difference in the size of one of two large *Eco*RI fragments of approximately 20 kb. The polymorphism is, however, easily detectable in a double digest with *Hin*dIII and *Eco*RI as illustrated in Figure 5. The calculated frequencies of the 4.6 kb and 5.2 kb *Hin*dIII alleles in the Asian population are 0.75 and 0.25, respectively. In the *Hin*dIII/*Eco*RI double digest the constant fragments have a length of 12 kb and 9 kb. The 4.6 kb *Hin*dIII fragment is cut in two smaller fragments of 3.1 kb and 1.5 kb when the polymorphic *Eco*RI site is present. We did not encounter *Eco*RI polymorphism in the fourteen 5.2 kb *Hin*dIII alleles studied. Based on these data the estimated frequency of the *Eco*RI site in this allele is less than 0.07. The polymorphic *Eco*RI site was encountered with a frequency of 0.12 in the thirty-four 4.6 kb *Hin*dIII alleles studied in the Asian population and the twenty-four alleles studied in the Caucasian population. Also the *Xba*I polymorphism originally detected in the Caucasian population did occur in the Asian population but with a slightly different allele frequency of 0.37 for the 1.5 kb allele and 0.63 for the 2.0 kb allele (Figure 6).

Discussion

Genetic heterogeneity in glycogenosis type II has been documented in extenso. Differences were established in the level of residual activity and the rate of enzyme synthesis in clinical variants (Mehler and DiMauro, 1977; Reuser et al., 1978, 1988; Reuser and Kroos, 1983; Shanske et al., 1986; Van der Ploeg et al., 1988, 1989a, 1989b; Hoefsloot et al., 1990d). Other studies disclosed a variety of defects in the posttranslational modification and in the intracellular transport of acid α-glucosidase in early and late onset forms of the disease (Reuser et al., 1985, 1987; Van der Ploeg et al., 1989a). Additional heterogeneity in glycogenosis type II was revealed as quantitative and qualitative abnormalities of acid α-glucosidase mRNA (Martiniuk et al., 1990b; Van der Horst et al., 1987; Hoefsloot et al., 1988; Reuser et al., 1988). Southern blot analysis of DNA from more than 20 patients did not result in the detection of gross insertions or deletions in the acid α-glucosidase gene (Martiniuk et al., 1990a, 1990b; Hoefsloot et al., 1988).

We show in this paper that the abnormal DNA restriction pattern detected in the past in a consanguineous Indian family from South Africa is not directly associated with lysosomal α-glucosidase deficiency. The unusual 5.2 kb *Hin*dIII allele was also found in two Taiwanese families with infantile glycogenosis type II. But in one of these families the disease co-segregated with the more common 4.6 kb instead of the 5.2 kb *Hin*dIII allele. In an extended study we discovered the 5.2 kb *Hin*dIII allele also among healthy individuals originating from mainland China, Japan and Indonesia. Apparently, we are dealing with a *Hin*dIII polymorphism in the Asian population which is very rare among Caucasians. The *Eco*RI and *Xba*I polymorphisms originally detected in the Caucasian population (Hoefsloot et al., 1990a, 1990b, 1991)

were also found in the Asian population with similar allele frequencies for each. This suggests that the *Hin*dIII polymorphism originated before the latter two.

Based on the exon-intron organization and the restriction map of the gene for acid α-glucosidase (Hoefsloot et al., 1990a) we conclude that the *Hin*dIII polymorphism must be due to an intronic insertion in the *Hin*dIII fragment spanning the exons 10 to 16. The insertion explains the abnormally large size of the BglII and StuI fragments in the DNA of the two South African patients (Van der Ploeg et al., 1989a). The polymorphic *Xba*I site is located in the *Xba*I fragment containing exons 2 and 3. The position of the polymorphic *Eco*RI site is exactly known i.e. in the intron between exon 14 and 15. The polymorphism is due to the variable presence of a T residue in the GAA(T)TC *Eco*RI recognition sequence (Hoefsloot et al., 1990a). Besides these polymorphisms resulting in altered restriction sites, a number of alternate base pairs have been reported to occur in cDNA and genomic clones (Martiniuk et al., 1990b; Hoefsloot et al., 1990a) Mutations leading to acid α-glucosidase deficiency have not yet been published, but the number of different mutations at the GAA locus is predictably high (Reuser et al., 1987, 1988).

With respect to the cause of clinical heterogeneity it is important to note that a complete deficiency of acid α-glucosidase mRNA has not been reported in late onset glycogenosis type II. This correlates with the expression of residual acid α-glucosidase activity in the adult form of this disease (Mehler and DiMauro, 1977; Reuser et al., 1978, 1983, 1987; Shanske et al., 1986; Van der Ploeg et al., 1987, 1988, 1989a; Hoefsloot et al., 1990d). The residual activity can be very low in exceptional cases but in general it amounts to 10-20 % of the normal level. In contrast, patients with a severe infantile form of glycogenosis type II have an invariably low activity of less than 2 %. This has led us to conclude that the clinical phenotype of glycogenosis type II is primarily determined by the level of functional enzyme. Conform this hypothesis an extremely low acid α-glucosidase activity was measured in the newly presented Taiwanese cases of infantile glycogenosis type II. The immunoblot analysis performed in both these cases revealed a similarly severe reduction in the amount of enzyme protein. But since the disease is linked to the 4.6 kb *Hin*dIII allele in one family and to the 5.2 kb allele in the other we have to presume that α-glucosidase deficiency is genetically heterogeneous in the Chinese/Taiwanese population as it is in the Caucasian population.

Materials and methods

Cell lines

Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 10 % fetal calf serum (Boehringer; Mannheim) and antibiotics, in an atmosphere of 90 % air and 10 % CO_2. The following cell lines were used: 86RD698 as control cell line derived from a healthy Caucasian; Cyst R and Cyst L, dermoid cysts of the GAA1 and GAA2 genotype (Swallow et al.,

1989); H.M., homozygote for the GAA2 allele (Swallow et al., 1989); 82RD167 and A.S., of patients with adult glycogenosis type II, and 217LAD and 266LAD, of patients with infantile glycogenosis type II, (Reuser et al., 1987), and 85-48 (Black), 78-14 and 81-1 (Indian), and 82-4 (Black), of patients from South Africa with infantile glycogenosis type II (Van der Ploeg et al., 1989a); R.F (father), R.M (mother) and R.P (affected child), and B.F (father), B.M (mother) and B.P (affected child) from two Taiwanese families with infantile glycogenosis type II.

Analysis of DNA and RNA

DNA was extracted from cultured fibroblasts and white blood cells of healthy individuals and patients with glycogenosis type II following standard procedures and digested with various restriction enzymes. DNA fragments were separated in 0.8 % agarose gels and transferred to nitrocellulose filters or Genescreen Plus membranes (Rigaud et al., 1987). RNA was isolated from cultured fibroblasts using the lithium chloride method (Auffray and Rougeon, 1980). RNA was size separated in 1 % agarose/formaldehyde gels and blotted onto nitrocellulose. Both DNA and RNA filters were hybridized with the radioactively labelled full-length acid α-glucosidase cDNA probe. (Sambrook et al., 1989; Feinberg and Vogelstein, 1983; Hoefsloot et al., 1988). The filters were washed to a stringency of 0.3 x SSC.

Enzyme analysis and immunoblotting

Acid α-glucosidase activity in cell lysates was measured with the artifical substrate 4-methylumbelliferyl-α-D-glucopyranoside or with the natural substrate glycogen as described before (Reuser et al., 1978). The protein concentration of samples was determined according to Lowry (1951). Immunoblotting was performed as described by Reuser et al. (1987).

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SUMMARY

Summary

The experimental work descibed in this thesis was undertaken to extend the knowledge on the structure and function of acid α-glucosidase, in relation to the lysosomal storage disorder glycogenosis type II. Chapter 1 gives a brief summary of recent literature on the intracellular sorting of proteins, and the targeting to specific organelles. Targeting signals are embedded in the structure of the protein, and some of these signals have been elucidated. Proteins with a lysosomal destination, like acid α-glucosidase, have an N-terminal signal peptide for entry into the lumen of the endoplasmic reticulum. They follow the secretory pathway till their sorting in the *trans*-Golgi reticulum. Most soluble lysosomal proteins are phosphorylated at mannose residues of the carbohydrate side chains. This enables them to bind to the mannose 6-phosphate receptor, and lysosomal targeting is accomplished. Lysosomal membrane proteins contain targeting signals in the cytoplasmic tail (Chapter 1).

The lysosomal storage diseases are introduced in Chapter 2. Interest in this group of diseases has started with the discovery of lysosomal α-glucosidase deficiency in glycogenosis type II. The primary defects of over thirty lysosomal storage diseases have been identified. The clinical heterogeneity within each of these diseases has been attributed to the occurrence of different mutant alleles. The primary amino acid sequences of a number of lysosomal proteins have been established in the past few years via cDNA cloning. Several mutations were identified in the genes of patients. Studying the types and the allelic combinations of the mutations in hexosaminidase A deficiency and in glucocerebrosidase deficiency has enabled the assessment of the genotype-phenotype relationship in Tay-Sachs disease and Gaucher disease, respectively. Other mutations have helped to determine the function of particular protein domains (Chapter 2).

The experimental work is introduced in Chapter 3, and focuses on the cloning and the characterization of the gene coding for lysosomal α-glucosidase. The knowledge is applied in studies on the cause of clinical heterogeneity among patients with glycogenosis type II. Chapter 4 describes the cloning of the cDNA coding for acid α-glucosidase and reports on the primary amino acid sequence. The precursor of acid α-glucosidase contains a predicted N-terminal signal peptide, which is lost soon after synthesis. Further, proteolytic trimming occurs at both the N- and C-terminal end of the precursor during intracellular transport. Seven potential glycosylation sites are indicated by the primary amino acid sequence of the precursor. Mature enzyme has only five of these sites. The sequence reveals a 40% amino acid identity with the non-lysosomal enzymes sucrase and isomaltase, pointing to a common evolutionary origin of the three enzymes (Chapter 4).

In Chapter 6 the isolation and the analysis of the acid α-glucosidase gene is

described. It consists of 20 exons, the first of which is non-coding. The boundaries of the promotor region are shown to be in accordance with the experimentally determined transcription-initiation site. The common evolutionary origin of acid α-glucosidase, sucrase and isomaltase may be reflected in a similar genome organization. However, the intron in the putative active site domain of acid α-glucosidase was not conserved in isomaltase.

Chapter 5 deals with the expression of acid α-glucosidase cDNA. cDNA constructs were made to obtain a clone with the complete coding sequence. The integrity of the cDNA is demonstrated by transfection of the constructs in COS cells. The post-translational modification and intracellular routing of the cDNA encoded enzyme are similar to acid α-glucosidase processing and transport in cultured human fibroblasts. But transfected COS cells were found to secrete large amounts of soluble precursor into the culture medium. Uptake of this precursor by enzyme-deficient cells from a patient with glycogenosis type II leads to degradation of stored glycogen, a definite proof that the cloned cDNA contains the correct coding information. In the course of these studies, a mutant cDNA was characterized. The acid α-glucosidase encoded by this cDNA is arrested in posttranslational modification and intracellular transport (Chapter 5).

In Chapter 7 the cloned cDNA is used as a probe to study the inheritance of mutant acid α-glucosidase alleles in an interesting family with glycogenosis type II. In this family both a mild (first generation) and a severe phenotype (third generation) of the disease were diagnosed. The patient in the first generation is demonstrated to be a genetic compound. One mutant allele of this patient codes for 30% residual acid α-glucosidase activity, whereas the second allele is silent. This latter allele is transmitted to the third generation where it combines with a second silent allele, giving rise to infantile glycogenosis type II (Chapter 7).

Chapter 8 reports on the heterogeneity of glycogenosis type II in the South African population. Two unrelated black patients are demonstrated to have a completely deficient synthesis of acid α-glucosidase and a total lack of mRNA (Chapter 9). Two patients in a consanguineous Indian family are shown to produce an apparently smaller than normal acid α-glucosidase precursor. The mutant allele in this family is characterized by an insertion in the acid α-glucosidase gene. But, a direct relation between the mutation and the insertion was excluded. It is shown in Chapter 9 that the insertion is a common polymorphism in the Asian population. Two other RFLP's (*Xba*I and *Eco*RI) are described in this Chapter. The studies described in this thesis have significantly extended the knowledge about the structure and the function of acid α-glucosidase. The newly acquired information might be useful for prenatal diagnosis and carrier detection in some specific families with glycogenosis type II. Eventually, the cloned acid α-glucosidase sequences may be applied for therapeutical purposes. Gene therapy can be envisaged, as well as large scale enzyme production for enzyme replacement therapy.

SAMENVATTING

Samenvatting

Het experimentele werk beschreven in dit proefschrift werd uitgevoerd teneinde meer kennis te verwerven over de structuur en functie van het lysosomal enzym zure α-glucosidase. Patienten met de lysosomale stapelingsziekte glycogenose type II hebben een deficientie van dit enzym. In Hoofdstuk 1 wordt een kort overzicht gegeven van de recente literatuur met betyrekking tot de routes die eiwitten intracellulair volgen naar de diverse organellen. De transportroute wordt gedicteerd door signalen die vastgelegd zijn in de aminozuur-volgorde van het eiwit. Een aantal van deze "postcodes" zijn bekend.

Eiwitten met een lysosomale bestemming, zoals zure α-glucosidase, hebben een signaal-peptide dat zorgt voor translocatie over de membraan van het endoplasmatisch reticulum. Ze volgen dezelfde intracellulaire route als de secretie-eiwitten, totdat de lysosomale route zich afsplitst in het *trans*-Golgi netwerk. Veruit de meeste oplosbare lysosomale enzymen wordt gefosforyleerd wanneer ze in het Golgi-complex arriveren. De gevormde mannose 6-fosfaatgroepen zijn het herkenningssignaal voor transport naar het lysosoom via de mannose 6-fosfaat receptor. Membraangebonden lysosomale eiwitten hebben daarentegen een herkenningssignaal in de cytoplasmatische staart (Hoofdstuk 1).

Het begrip "lysosomale stapelingsziekte" wordt geïntroduceerd in Hoofdstuk 2. De ontdekking van lysosomaal α-glucosidase-deficiëntie bij glycogenose type II heeft het onderzoek naar de oorzaak van deze groep van ziekten gestimuleerd. Van meer dan dertig lysosomale stapelingsziekten is nu het primaire defect bekend. De klinische heterogeniteit die elk van deze ziekten kenmerkt, wordt toegeschreven aan het voorkomen van verschillende mutante allelen van hetzelfde gen. In de afgelopen jaren is de primaire aminozuur-volgorde van een aantal lysosomale eiwitten opgehelderd via cDNA-klonering. Ook werden de exacte mutaties bepaald in het DNA van patiënten. Door te kijken welke combinaties van mutaties voorkomen in de verschillende subtypen kunnen nu langzaamaan relaties gelegd gaan worden tussen genotype en fenotype. Als voorbeeld worden in Hoofdstuk 2 de ziekte van Tay-Sachs (hexosaminidase deficiëntie) en de ziekte van Gaucher (glucocerebrosidase deficiëntie) besproken. Andere mutaties hebben door hun positie informatie over de functie van eiwit-domeinen opgeleverd.

Het experimentele werk in dit proefschrift wordt ingeleid in Hoofdstuk 3 en betreft de klonering en de karakterisering van het gen dat codeert voor lysosomaal α-glucosidase. De kennis wordt toegepast bij het zoeken naar de oorzaak van klinische heterogeniteit in glycogenose type II. Hoofdstuk 4 beschrijft de klonering van het zure α-glucosidase cDNA, en de afgeleide aminozuur-volgorde. Lysosomaal α-glucosidase blijkt, zoals verwacht, een signaalpeptide te hebben voor import in het

endoplasmatisch reticulum. Na synthese wordt het lysosomaal α-glucosidase ingekort door het afsplitsen van peptiden aan beide einden. Het enzym blijkt verder zeven potentiële glycosyleringsplaatsen te hebben. 40% van de aminozuren van α-glucosidase is identiek aan die van sucrase en isomaltase, twee enzymen die uitsluitend in de darm voorkomen. Dit wijst erop, dat deze drie enzymen van hetzelfde oer-gen afgeleid zijn (Hoofdstuk 4).

In Hoofdstuk 6 wordt de isolatie van het lysosomaal α-glucosidase gen beschreven. Het gen, dat gelegen is op chromosoom 17, bestaat uit 20 exonen, waarvan het eerste geen coderende sequenties bevat. De plaats waar de RNA-transcriptie start werd bepaald, en de lengte van het promoter-gebied werd vastgesteld. Sequentie-analyse wijst uit dat lysosomaal α-glucosidase een typische "huishoud-gen" promoter heeft.

Omdat lysosomaal α-glucosidase, sucrase en isomaltase een gezamenlijke evolutionaire oorsprong hebben, zou het kunnen zijn dat de genomische organisatie van de genen vergelijkbaar is. Hiervoor werden echter geen aanwijzingen gevonden. Een intron in de veronderstelde katalytische site van het lysosomaal α-glucosidase kon niet worden aangetoond in isomaltase (Hoofdstuk 6).

Om een cDNA-kloon te verkrijgen die voor het complete lysosomaal α-glucosidase codeert, werden er verschillende partiële cDNA's aan elkaar gekoppeld. Vervolgens werd de integriteit van dit cDNA-construct getest, door het tot expressie te brengen in apeniercellen. De post-translationele modificaties en de intracellulaire transport-routes van het cDNA gecodeerde enzym waren vergelijkbaar met die van het lysosomaal α-glucosidase dat gesynthetiseerd wordt in gekweekte humane fibroblasten. Een deel van het door de apeniercellen geproduceerde enzym wordt gesecreteerd. Opname van dit enzym door lysosomaal α-glucosidase-deficiënte cellen van een patiënt met glycogenose type II leidt tot de degradatie van het in de lysosomen gestapelde glycogeen. Met deze experimenten werd de integriteit van het gekloneerde cDNA aangetoond. In bepaalde cDNA constructen werd een punt-mutatie ontdekt, die het transport van lysosomaal α-glucosidase naar het lysosoom blokkeert (Hoofdstuk 5).

In Hoofdstuk 7 wordt het gekloneerde cDNA gebruikt als probe om de overerving van mutante lysosomaal α-glucosidase-allelen te bestuderen in een familie met glycogenose type II. Het bijzondere van deze familie is, dat er twee patiënten in voorkomen; één met een milde vorm van de ziekte (eerste generatie) en één met een ernstige vorm (derde generatie). Met een combinatie van methoden wordt aangetoond dat de patiënt in de eerste generatie twee verschillende lysosomaal α-glucosidase-allelen heeft. Eén allel zorgt voor 30% rest-aktiviteit; het andere allel draagt niet bij aan de totale aktiviteit. Dit laatste allel wordt doorgegeven aan de patiënt in de derde generatie, die behalve dit allel nog een tweede, soortgelijk allel heeft. Deze combinatie van allelen leidt tot de zeer ernstige vorm van glycogenose

type II.

Hoofdstuk 8 rapporteert over de heterogeniteit van glycogenose type II in de Zuid-Afrikaanse populatie. Bij twee niet-gerelateerde (negroïde) patiënten wordt een complete afwezigheid van lysosomaal α-glucosidase eiwit en mRNA (Hoofdstuk 9) aangetoond. Bij twee Indiase patiënten uit een consanguin huwelijk lijkt het lysosomaal α-glucosidase kleiner dan normaal. In deze familie wordt het mutante allel gekenmerkt door een insertie van 0.5 kb. Een directe relatie tussen de mutatie en de insertie blijkt er echter niet te zijn. In Hoofdstuk 9 wordt aangetoond dat de insertie een vaak voorkomend polymorfisme is in de Aziatische populatie. In dit zelfde hoofstuk worden nog twee andere RFLP's (voor *Xba*I en *Eco*RI) beschreven. De studies beschreven in dit proefschrift hebben veel nieuwe informatie opgeleverd omtrent de structuur en de functie van het lysosomale α-glucosidase. Deze kennis kan van nut zijn voor prenatale diagnostiek en dragerschap-onderzoek, wanneer in bepaalde gevallen enzym-diagnostiek geen uitsluitsel geeft. In de toekomst kunnen de gekloneerde sequenties mogelijk gebruikt worden voor de grootschalige produktie van lysosomale α-glucosidase voor enzymtherapie, of voor gentherapie.

Curriculum vitae

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Nawoord

Dit proefschrift had niet tot stand kunnen komen zonder de steun van velen. Allereerst mijn promotor, professor Galjaard, die mij de gelegenheid heeft gegeven dit onderzoek uit te voeren. Uw positieve reacties op de resultaten ervan heb ik als zeer stimulerend ervaren.

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